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BIOLOGICAL CONSEQUENCES OF PLANT RESIDUE DECOMPOSITION IN SOIL

W. C. Snyder, et al

California University

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BIOLOGICAL CONSEQUENCES OF PLANT RESIDUE DECOMPOSITION IN SOIL

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ABSTRACT

This 7-year study reports on the nature and action of phytotoxic substances that are released to the soil from decomposing plant residue. Both the favorable and unfavorable effects were observed. Decomposition products in field soils were analyzed and some of the more active ones were chemically defined. The toxicities of these products on seedlings and roots of field-grown crops were tested under such varying conditions as time, moisture, aeration, temperature. The effects of these residues on plant pathogens were also observed, and the role of these organic residues on the epidemiology of seedlings was determined.

FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. This focus is provided by the multidisciplinary programs of the National Environmental Research Centers, which are engaged in

- studies on the effects of environmental contaminants on man and the biosphere
- a search for ways to prevent contamination and recycle valuable resources.

Disposing of agricultural waste, which is a substantial amount of all solid waste, is a matter of public concern. As part of its research efforts in this area, the Solid Waste Research Laboratory of the National Environmental Research Center in Cincinnati funds projects researching many different approaches, as well as projects designed to determine the effects resulting from these approaches. The facts and observations from this publication should help develop improved means of waste disposal; the results are also important to the culture of agricultural crops because incorporating organic substances may have a profound effect on plant disease.

ANDREW W. BREIDENBACH, Ph.D.
Director, National Environmental
Research Center, Cincinnati

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INTRODUCTION

Plant material returned to the soil represents the primary source of substrate for soil micro-organisms. In arable soils the plant material takes the form of crop-remains or organic amendments which may be incorporated into the soil during normal agronomic practices. Incorporation of such large amounts of substrate is an important event in the microbiology of the soil. A succession of soil micro-organisms colonizes and utilizes the residue in the degradative process producing a succession of organic compounds with different degrees of biological activity. Similarly, a major component in solid wastes consists of organic matter which is of plant origin. Disposal and recycling of the enormous quantities of these materials with minimal ecological disturbance presents problems both serious and complex.

Plant residues and the resulting decomposition products have long been considered as having important effects on all phases of plant development including soil productivity, severity of root disease, and biological control of soil-borne diseases. As with most problems associated with soil, the results obtained by the various investigators have been frustratingly unpredictable. Anyone familiar with this area of research knows that even though the ecology of organic residue degradation has long been studied, the results are commonly varied, difficult to interpret, or are open to controversial interpretation. Soil type alone, as well as the environment, exert a tremendous effect in such studies. In some instances benefits to plants are derived while in others the effects are detrimental. In some cases the damage has been attributed to phytotoxic compounds, both constitutive materials in the organic residue and decomposition products of the residue. In other cases depletion of available nitrogen and phosphorus,

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anaerobiosis, and toxic concentrations of reduced ions, toxic salinity levels, pathogenic fungi and combinations of these factors have been implicated. Many critical questions remain conflicting including problems of accumulation, stability, persistence and concentrations of these compounds in soil. There is still considerable doubt as to how many of these compounds are actually formed under field conditions and accumulate in sufficient quantities to produce their effects. *There are also* questions regarding the chemical nature of the phytotoxic compounds, their specific effect on plants in the absence of other casual factors and their overall ecological significance.

During the past six years we have conducted a detailed study of the basic aspects of the problem of the biological consequences of crop residue decomposition in soil in order to resolve some of the more important questions mentioned above. Analyses were made of the decomposition products in field soils, and some of the more active ones were chemically defined. Toxicities of these products to seedlings and to roots of field grown crops were tested using many different organic residues under different moisture levels, timing, and conditions of aeration. The effect of crop residues on the behavior of soil-borne plant pathogens were observed and the role of these organic residues upon the epidemiology of seedling diseases followed. Both favorable and unfavorable effects of crop residues in agriculture were demonstrated and analyzed.

It is believed that the facts and observations recorded here will help materially in the development of improved means of waste disposal in general. Also, results are of particular importance to the culture of agricultural crops since the incorporation of organic substances may have a profound effect upon plant disease. This report summarizes some of the findings obtained in these studies. For a more detailed account the reader is directed to the references listed on page

An important side benefit of the study has been the training of young scientists. Graduate students and postdoctoral assistants gained invaluable experience in biological research in the process of contributing their considerable interest, energy and enthusiasm to the program. Furthermore, the conferences and frequent discussions held locally and regionally in connection with the research fostered cooperation with an exchange of information.

DECOMPOSITION OF CROP RESIDUES IN SOIL AND FORMATION OF PHYTOTOXIC PRODUCTS.

One of the important questions associated with crop residues is whether substances possessing phytotoxic properties are formed during their decomposition. This aspect was investigated extensively in field and laboratory experiments.

Experimental Techniques for Obtaining Decomposition Products and Bioassay of their Activities.

The plant-residue-containing soils were obtained in the Salinas Valley, California, from fields treated in the conventional way by growers. In some fields, lush cover crops of barley, rye, or wheat with or without vetch, just before full head and averaging 10-15 T/acre of green plant material, had been disced or plowed under. In others, broadbeans, Sudan grass, or remnants of a commercial crop of broccoli had been similarly incorporated. The soil usually was clay loam, pH 7.2-8.0.

Sampling was begun in 16 fields during the first week of December. In some of them, the crop had just been turned under; in others, the plant residue had been decomposing for up to 25 days. Sampling was repeated 4 times at 10-day intervals. In the first week in March, a second series of samplings was begun in 14 other fields and repeated 4 times on a 10-day schedule. The temperature of the soil during the December-January samplings and those of March-April fluctuated between 32° and 55° F, respectively. At each sampling period, composite samples of plant residue with the surrounding soil were collected in plastic bags. These were taken to the laboratory and extractions carried out usually within 1-3 days. The samples in the meantime were kept either cold or frozen to ensure minimum change in the decomposition state of the products.

Extraction procedures. In the first series of extractions, to determine which component, the soil or the plant residue, contained the phytotoxic fraction, each sample was subdivided into 3 components: 1) soil and residue in the relative proportions found in the field; 2) soil, after all recognizable plant residues had first been removed; and 3) plant residue free from soil. Cold-water extracts were obtained from each and tested for phytotoxicity. In most of the later experiments, because the first 2 components showed low phytotoxic activity, extracts were made only of the decomposing plant residues. This was done by first picking out the individual pieces of recognizable plant residue from the soil. Most of the soil adhering to the residue was brushed off to ensure minimum loss of any water-soluble decomposition products; the residue then was cut into sections approximately 1/2-1 cm long, placed in beakers containing distilled or deionized water, and extracted for 12 hr with regular stirring in the cold (50° F). Thirty g of plant material (fresh weight) or 15 g (oven-dry weight) were extracted with 150 ml of water. The material was strained through cheesecloth, the solids discarded, and the liquid centrifuged at 2,000 rpm for 20 min. The sediment, made up largely of soil in close contact with the plant residue and the smaller fragments of plant debris, was saved for further tests. The supernatant liquid was decanted and centrifuged again in a Spinco refrigerated ultracentrifuge at 35,000 rpm for 30 min. This procedure gave a clear supernatant liquid virtually free of bacteria and fungi; no further filtration was necessary. The pH and relative amounts of soluble salts in the extracts then were determined. The tests for soluble salts were based on electrical conductivity measurements carried out with a solubridge as described in the U.S.D.A. Agriculture Handbook No. 60. If the conductivity of the extract gave a reading above 1.8 millimhos/cm at 25° C, the extract was dialyzed against running tap water for 2-4 hr to reduce the soluble salt content. Due to some loss of phytotoxicity after the dialysis, however, the extracts in most tests were split into 2 portions, one of which then was extracted with ether. In such instances, 600 ml of ether were used for each 100 ml of solution and each extract was extracted 3 times with 200 ml ether at each extraction. The ether solution, floated on 15 ml of distilled water, was removed by evaporation in a water bath at 45° C under nitrogen. The aqueous solution which remained was used directly or diluted before testing.

Tests for phytotoxicity. The main bioassay tests used for evaluating the relative phytotoxicity of the various extracts were based on inhibition of germination of lettuce seed and seedlings. As additional tests, seed and seedlings of broccoli, white beans, and tobacco also were used. In the tests, 6.5-ml portions of the extract to be tested were added to petri dishes containing 2 layers of Whatman No. 1 paper. After the solution had been absorbed by the filter papers, 50 lettuce seeds (var. Great Lakes or of the other seeds mentioned above) were spread evenly on the paper. The dishes were placed in partially covered boxes and incubated at room temperature (78-75° F) for 96-120 hr. Sterile water, containing NaCl, $CaCl_2$, $MgSO_4$, and $NaSO_4$ salts in equal proportions and adjusted to give electrical conductivity readings of 1.5-1.8 millimhos/cm at 25° C, was used in the controls in place of the various extracts. Extracts of soils without the plant residues, but likewise adjusted to give conductivity readings of 1.5-1.8 millimhos/cm, also were used as controls. Tests with each extract were replicated 3 or 4 times.

Twenty seedlings, selected at random, were removed from each dish after the proper incubation period and their primary roots (radicles) measured; and, by comparison with controls, relative phytotoxicity of the various extracts was evaluated. In this test, referred to here as the lettuce bioassay, the initial seed-germination phase and the growth of the radicle which takes place in the 96-120 hr test period are combined in the readings. No attempt was made to separate the 2 phases. This made the tests more convenient to carry out, confusion in separating the 2 phases was avoided, and differences between the effects produced by the various extracts were more readily apparent.

Association of Root Injury of Field-grown Lettuce and Spinach Seedlings with Decomposing Plant Debris.

Detailed observations were made on lettuce and spinach plants growing in the fields to determine whether the stunting, uneven growth, and root injury often observed in the Salinas Valley, especially during the early seedling growth, could be partly attributed to toxic decomposition products. Fields in which lettuce and spinach, at stages of growth from just germinating to where leaves were 2-3 in. long, were examined. Small trenches were carefully dug around the seedlings, exposing much of the root system. Considerable quantities of plant debris at various stages of decomposition were present in most of the fields, with the roots of the seedlings closely intermingled with the debris. When a root was growing in contact with or close proximity to a fragment of decomposing plant debris, a discolored or sunken lesion often was found on that region of the root. There also were many instances of browning of the apical meristems and other radicle injury, characteristic of injury found in the laboratory; and again, these were found near fragments of decomposing plant debris. Many seedlings with the surrounding soil were carefully dug up. When the soil was gently washed away, many pieces of decomposing plant debris were found with the roots. Many discrete and isolated lesions were found on the roots, often in association with debris fragments. When isolations were made from such lesions, no known single pathogen was consistently obtained. The organisms most frequently found were common soil saprophytes.

Further, plant debris was not evenly distributed through the soil, often occurring in clumps of varying size. There were, however, few areas of the soil where the roots would not encounter at least a few fragments of plant debris. Seedlings growing in proximity to large clumps of plant debris were usually stunted, although exceptions were found.

Attempts were made to evaluate the relative abundance of plant-residue fragments found in some of the fields. From such observations, it was hoped that some indication could be obtained of the relative frequency and concentration of toxic material that the seedling roots may encounter in the field. For this purpose, 2-, 3- and 4-in. soil cubes were removed from various fields. All recognizable plant-residue fragments were picked out and weighed. There were wide variations in the results, but on the average there were 4-7 g of residue (fresh weight) in each 100 g of moist soil. There also were 90-165 plant-residue fragments over 1 in. long in such a sample. Because of the variability between samples, locations, and fields, no conclusions could be drawn except that some seedlin roots might encounter many fragments of decomposing plant debris and others might encounter relatively few.

Greenhouse Experiments with Plant Residue and Soil from the Field.

Following the turning under of the previous crop, the usual practice in the Salinas Valley in the growing of lettuce or other row crops is to hill the soil into double-row beds and plant the seed in 2 rows, 1 on each side of the bed. The soil is kept moist by furrow or sprinkle irrigation until seeds germinate and seedlings begin to grow. Water then is applied as needed. In many instances, the seed is planted only a few days after the previous crop has been turned under; at other times, the fields are not seeded for 1-5 weeks. If no rain occurs during this time (as in this study), the decomposition then begins when the seed is planted and irrigation is applied. Such conditions are ideal for the formation of toxic products, especially if there was continuous moisture during decomposition of the residue. Instances of poor germination and seedling growth were reported by some of the growers in the Salinas Valley in instances where lettuce was planted too soon after turning under barley or rye cover crops. Therefore, experiments were undertaken to determine whether severe phytotoxicity could be detected if the above practice was repeated in the greenhouse with soil and plant residue obtained directly from the field.

Two fields were selected: in one, barley had been turned under 2 weeks previously; in the other, rye had been treated in the same manner. Composite soil samples were obtained from these fields, mixed thoroughly, and placed in pots. As controls, similar soil from which all plant residue had been removed was used. The pots were divided into 2 series, each replicated 4 times. In one series, the soil was kept wet continuously; in the other, it was allowed to dry out before additional water helped maintain a more even temperature and moisture, and were kept on a bench outdoors (day, 60-70° F, night 50-55° F). After 8 days, 40 lettuce seeds were planted in each pot. All soils were watered normally after this time. After 16 days, 20 seedlings were carefully removed from each pot and their roots examined and measured. Stunting and other root injury were observed in seedlings from the pots containing soil and plant residues kept wet prior to planting. For example, in this series, the average root length of lettuce seedlings in the pots containing the barley residue was 13 mm.

Where rye residue was present, the roots averaged 15 mm. In the comparable series kept relatively dry prior to planting, the lettuce roots in pots containing barley residue were 22 mm and were 25 mm where rye was used. The tap root was severely stunted, often with brown and dead meristems. No pathogens were isolated from such roots. In the controls (without the decomposing residue), the roots appeared white and healthy and averaged 31 mm long; only a slight reduction (28 mm) was observed in those removed from the wet series. Further, although only a few seedlings in the controls produced secondary roots, those growing in pots containing the decomposing residues produced 2-5 secondary roots at the crown. The hypocotyl region of the seedlings did not seem affected at this time and averaged 55-60 mm in all treatments. There was no recovery and when the experiments were discontinued after 50 days, there was a marked reduction in the fresh weight of plants. For example, the average fresh weight of the lettuce plants growing in pots containing the decomposing barley residues was 4.9 g; and of those containing rye, 8.1 g. The average for the controls was 11.4 g.

Bioassays with Seed and Seedlings Show the Presence of Phytotoxic Substances in Aqueous Extracts of Plant Residues Decomposing in the Field.

Since field and greenhouse observations indicated that poor growth and injury to plant roots were often associated with decomposing plant residues, experiments were undertaken to determine whether phytotoxic substances could be extracted from such residues and their activity assayed.

Results showed considerable differences in effects produced by the various types of extracts on germination and growth of lettuce. In general, no marked adverse effects on lettuce were obtained with extracts of soils from which all recognizable plant residue had been removed prior to extraction or those in which the soil and plant residue were in the proportions found in the field. In most instances, some stimulation was obtained with extracts of plant residues only when such residues had been freed of most of the soil prior to extraction. The amount of toxicity also varied with the decomposition period and type of plant residue present in the soil. In general, however, highest activity was exhibited by extracts obtained during the early stages of decomposition.

A few tests also were carried out to determine whether toxic substances could be extracted from the clumps of plant residue often found in field soil. This could be a very important factor in many fields since, during plowing or discing of a cover crop, the plant residue is not evenly distributed in the soil and many instances occur where relatively large quantities of residue are clumped together. Water extracts of many of these clumps inhibited germination and growth of lettuce. The toxicity was most pronounced in clumps of residue in which relatively little soil was present. As the proportion of soil to residue was increased, the toxicity declined. With the extraction procedures and bioassay tests used, toxic substances were only extracted from clumps of residue in which the percentage of soil did not exceed 80% fresh weight. These observations could have an important bearing on stunted growth of lettuce or other seedlings often observed in the field, particularly where stunting is unevenly distributed.

Soil in Direct Contact with Decomposing Plant Residue Toxic to Lettuce.

Experiments also were undertaken to determine whether the layer of soil in direct contact with plant-residue fragments might at times be phytotoxic. In such tests, the soil adhering to the residue was carefully washed in a minimum quantity of water, concentrated by centrifugation at 2,000 rpm, placed in petri plates, and covered with a layer of filter paper pressed tightly against the soil to eliminate air bubbles. Soils from the same sample, but not in the immediate vicinity of plant residues were prepared in a similar manner as controls. Lettuce bioassays were carried out in the regular manner. As a comparison, the activity of the water extracts of the same residues from which the soils were obtained also were tested. Germination and growth of lettuce was inhibited by soil obtained from the surface of decomposing plant residue and very little or no inhibiting activity was shown by soil not in immediate contact with the residue. The results of these tests, however, are not entirely conclusive, since a certain amount of the toxic decomposition products could have come from the plant-residue fragments during the initial washing procedure.

The toxicity of the soil in contact with the plant residue was markedly reduced by 3 washings with water and toxic activity could be detected only in the first wash water. Toxicity of this soil also was reduced by allowing it to stand in petri dishes for 2-4 days at room temperature. This was probably due to microbial activity, since considerable fungal and bacterial growth was evident at this time.

Bioassays with Lettuce Reveal Presence of Phytotoxins in Decomposition Products from Barley Residues.

Sequential studies were carried out in a number of fields to determine the relative frequency and degree of the more common crops grown in the Salinas Valley. In this series of experiments, only extracts of decomposing plant residues freed from the soil prior to extraction were tested. The results were based on lettuce bioassays and were described as percentage inhibition or stimulation when compared with controls.

The activity of the aqueous extracts of residue obtained from 6 different fields in which barley had been decomposing for various periods of time differed considerably. The degree of toxicity seemed to depend largely on the length of the decomposition period. The duration of the toxic period also varied considerably among the different fields. Some phytotoxicity was obtained with aqueous extracts of undecomposed barley residues. In general, however, substances with the most toxic properties were most frequently obtained when the barley plant material had been decomposing in the soil for approximately 10-25 days. Such extracts inhibited germination and growth of lettuce by 30-70%. Following this period, the toxicity declined and stimulatory effects were frequently obtained. In some fields, the peak of toxicity was reached relatively quickly, by the tenth-fifteenth day; declined rapidly; and by the twenty-fifth day, little or no toxicity could be detected. In others, peak of toxicity was often not reached until the twentieth to twenty-fifth day and this was usually followed by a rapid decline.

Lettuce Bioassays Show that Decomposition Products from Rye Residues May Be Phytotoxic.

Considerably more variability in relative toxicity was exhibited by the aqueous extracts of residue obtained from fields in which rye had been turned under. In general, however, the results were essentially similar to those obtained with barley. Extracts with marked phytotoxicity were obtained when rye residues had been decomposing for 10-25 days and the toxicity declined as the decomposition period increased, until by the thirtieth day little or no toxicity was evident.

Bioassays Reveal Presence of Phytotoxic Substances in Decomposition Products from Wheat, Vetch, Sudan Grass, Broccoli, and Broadbean.

Results essentially similar to those described above were obtained in fields where these 5 crops were grown. Decomposition products of vetch and Sudan grass, however, were only weakly toxic. Extracts from both broccoli and broadbean showed marked phytotoxic activity at 0-1 days--at a time when very little decomposition could have taken place. This suggests that phytotoxic compounds may be present in the normal tissues of these plants. Again, toxicity declined with increasing period of decomposition and was negligible thirtieth day.

Other Injurious Effects Produced by Toxic Decomposition Products.

In addition to inhibiting germination and growth of lettuce, other injurious effects were produced by these various extracts on lettuce, tobacco, white beans, and broccoli seed and seedlings. Some of the most active extracts, for example, completely inhibited germination of these seeds; the lettuce seeds often swelled and became dark. When the seeds were removed from the toxic solutions, washed, and placed in sterile water, they did not germinate, in many instances; and apparently had been killed. Seed exposed to other extracts and similarly treated eventually germinated, but the seedlings grew abnormally. Many of the seeds exposed to relatively nontoxic extracts germinated erratically and unevenly; some, even in the same petri dish, germinated well and grew normally, whereas, with others, germination was delayed or inhibited. No such abnormal results were obtained in the controls.

Marked Sensitivity of Primary Root to Toxic Decomposition Products.

One of the more striking effects observed was sensitivity of the primary root or radicle to the toxic decomposition products. In many of the extracts, germination took place relatively normally, but the growth of the radicle was either completely or partially inhibited. Often, the hypocotyl was only slightly affected. The apical meristem region became discolored and apparently was killed. In beans, the meristem region of the radicle became necrotic; then, numerous secondary roots were put out, some of which remained uninjured. When young seedlings were used, visible injury to the apical meristem region of the primary root occurred less then 2 hr after exposure to toxins. With tobacco seedlings, the apical meristems turned dark brown; in lettuce, the browning was less pronounced; and in bean seedlings, the meristems turned gray. When bean seedlings were placed in some of the more toxic extracts, wilting occurred in 3-10 hr and the roots often turned brown.

In other tests, small drops of the more toxic extracts were placed on roots of lettuce or bean. Injury in the form of depressions and discoloration were noted in such spots in less than 1 hr; and often, if the root was small, the region of the root under the drop collapsed and shriveled.

Effect of Salinity on Relative Toxicity of Extracts.

As mentioned earlier, many of the extracts showed high electrical conductivity values, usually associated with high salt concentrations. Therefore, various precautions were taken to ensure that the injurious effects of salinity were not confused with phytotoxicity caused by organic substances formed during the decomposition of plant residues. Since soil salinity is a serious problem in most of the semi-arid areas, including the Salinas Valley, attempts also were made to determine whether phytotoxicity exhibited by the various organic decomposition products could be affected by salinity.

Salinity effects are mostly negligible in extracts with electrical conductivity readings of 2.0 millimhos/cm or less. Electrical conductivity of all extracts, therefore, was determined prior to bioassays. Extracts having values above 2.0 were either dialyzed, diluted, or re-extracted with ether. Thus, bioassays were carried out only with extracts with conductivity values of 1.5-1.8 millimhos/cm and were compared with controls having similar values. Additional tests were carried out with known nontoxic solutions, the salinity of which had previously been adjusted to various values; these were compared with known toxic solutions having similar conductivity readings. No appreciable effects on germination and growth of lettuce or other test plants were detected in the nontoxic solutions having conductivity values of up to 3.0 millimhos/cm. The roots of the seedlings remained white in such solutions. Some delay in germination and inhibition of root growth were obtained at the higher conductivity values, but there was complete recovery when salinity was reduced.

Most of the toxic and nontoxic extracts were re-extracted with ether. The electrical conductivity of such extracts was 0.01-0.08 millimhos/cm. When bioassays were repeated with such extracts, essentially similar results to those described earlier were obtained. These and other tests indicate that the phytotoxic effects produced on the lettuce and other test plants were primarily due to toxic organic substances formed during the decomposition of the various plant residues in the soil.

It was noted, however, that increased salinity would enhance phytotoxicity. For example, whereas fair germination and growth of lettuce were obtained in water or soil extracts with conductivity readings of 4.0-5.0 millimhos/cm, very little growth was obtained in extracts of decomposing plant residue with values of 2.0 millimhos/cm and no germination in those in which conductivity was adjusted to 3.0 millimhos/cm. Similarly, there was little or no recovery when such seeds or seedlings were returned to nontoxic solutions. Further, in some of the weakly toxic water or ether extracts--i.e., those producing inhibition of 10-20% in the bioassay tests--50-60% inhibition occurred when salinity of the extracts was raised to conductivity values of 2.0-2.5 millimhos/cm. The browning of apical meristems, root stunting, and other injurious effects were more severe and were produced more rapidly at the higher salinities.

During the extractions, the electrical conductivity of the different extracts varied widely (1-5 millimhos/cm). The conductivity was usually highest in extracts of plant residue that had been decomposing in the soil 5-15 days. As decomposition progressed, the conductivity values usually declined and by the twenty-fifth to thirty-fifth day were similar to extracts of the saturation paste of the surrounding soil.

Saturation extracts of soil which contained decomposing plant debris gave higher conductivity values than the saturation extracts of the same soil from which most of the debris had been removed. Since most plants were more sensitive to salinity during the germination and seedling stages, a few tests were carried out to determine whether soil salinity might increase during decomposition of plant residue. For this purpose, 2 soils were used; field soil in which the conductivity of the saturation paste extract was 1.1; and a nonagricultural soil in which the conductivity was 0.2 millimhos/cm. Various amounts of partially green barley straw were added and allowed to decompose for 23 days. The conductivity of saturation paste extracts was obtained in the usual manner at 0- and 23-day decomposition. Salinity of the soil was markedly increased in soils containing decomposing plant residues. For example, the electrical conductivity of extracts of field soil to which 2% and 5% barley residues had been added increased to 1.7 and 4.5 millimhos/cm, respectively, after 23 days of decomposition. In the nonagricultural soil, with low initial salinity, the addition of 2, 5, and 10% barley residues increased the electrical conductivity of the extracts to 1.7, 3.0, and 4.0 millimhos/cm after the 23-day decomposition period. The results suggest that the salts accumulated in the tissues of the plants during growth might be released when the tissues decompose. If large amounts of plant debris are present and leaching does not take place rapidly, salinity of the surface layers of the soil might be appreciably increased. Thus, in addition to the toxic organic substances formed during the decompositon of plant residues, phytotoxicity may be enhanced by the increased salinity. More study of this phase is required, however, before full evaluation of the importance of this phenomenon under field conditions can be made.

IDENTIFICATION OF SUBSTANCES PRODUCED DURING PLANT RESIDUE DECOMPOSITION IN SOIL.

The widespread occurrence of phytotoxins in nature and their apparent involvement in plant damage and root diseases suggests their over-all importance in the biology of soil. Information on their production, activity, and chemical nature is therefore highly desirable. This portion of the report deals with the identification of phytotoxic compounds formed during decomposition of plant residue in soil. Although it is impossible to identify all of these materials the characterization of those involved in the effects of residues investigated in this project is necessary. Without this information the relative importance of these compounds cannot be assessed.

Production of Phytotoxins and Partial Purification.

Most of the methods used to obtain and assay phytotoxic extracts have been described previously. Under conditions optimum for their formation, phytotoxins could be detected in the field within 5-10 days after the green cover crop had been turned under. Maximum toxicity was reached within approximately 25 days, after which phytotoxicity declined. This pattern, however, varied from field to field and even within the same field. To obviate some of these fluctuations and to ensure a constant supply, the major source of the toxic extracts in the present work was from plant material decomposing in the laboratory. However, extracts from material decomposing in the field were used to verify the laboratory findings.

The laboratory decomposition procedure employed was as follows: 100 g (fresh weight 9 of approximately 1-month-old, field-grown green barley plants of 1-month-old, greenhouse-grown cotton, cowpea, or soybean were mixed with 300 g of air-dried field soil and placed in beakers or earthenware crocks; 150 ml of water were added and the mixtures were incubated for periods up to 2 months. Extracts were obtained at weekly intervals. To obtain the extracts, 150 ml of water were added to the mixture and stirred; after 1 hr the supernatant of the mixture was decanted, centrifuged to remove soil and plant debris, filtered, concentrated to one-tenth its volume under reduced pressure, and centrifuged at 7,000 rpm. The supernatant was then decanted and frozen until needed.

Experiments revealed that temperatures from 16 to 24 C had little effect on phytotoxin production. Therefore, the decomposition experiments were conducted at room temperature (approximately 23 C). Moisture content of the soil-residue mixture, on the other hand, had a pronounced effect on phytotoxin production. At a moisture content of 30% (of the dry weight of the soil-residue mixture) and below (moisture retention of the soil at one-third atmosphere was 20.2%), extracts had stimulatory properties. Above this moisture content, highly phytotoxic extracts were obtained.

The relative phytotoxicity of these extracts was determined by means of bioassays, usually with tobacco seeds, as described previously. For the bioassays, extracts were diluted with water to their original concentration. Toxin production with time under laboratory conditions was similar to that obtained from barley residues decomposing in the field. Extracts were usually first toxic 7-10 days after incorporation of the barley into the soil. Maximum toxicity occurred in about 3 weeks, remained high until 6 or 7 weeks, and then declined.

As previously indicated, extracts had high electrical conductivity, a characteristic of high salt concentrations. Such a high concentration of electrolytes interfered with the bioassays. The extracts, therefore, were dialyzed against running tap water for 1 hr or until their conductivity was reduced to less than 2.0 millimhos/cm. In general, the activity of the dialyzed extracts was reduced by 5-10%. Phytotoxicity was lost, however, after 5 hr of dialysis.

The crude aqueous extracts of the decomposing plant organic matter were first adjusted to pH 2.0 with 1 N HCl and then extracted three times with peroxide-free diethyl ether (1:1, v/v). The ether-soluble fraction was washed three times with 5% aqueous $NaHCO_3$; the aqueous layer was retained and acidified with 1 N HCl to pH 2.0 and subjected to extraction three times with ether. The ether-soluble fraction was dried over $CaCl_2$ and evaporated. The product obtained was a brown viscous liquid with a pungent odor. Little or no phytotoxic materials were obtained when the crude aqueous solution was extracted with ether at pH 10.0. The brown residue, when diluted with water to the volume of the original crude extracts, was found to be highly phytotoxic. The phytotoxicities of ether extracts and the parent crude nondialyzed extracts were compared by means of a dilution series. These experiments revealed that the phytotoxicity of the ether-soluble fraction was approximately 60% of that of the crude parent extracts. The remaining phytotoxicity may represent salts not extractable in ether.

Identification of Ether Soluble Phytotoxins by Gas and Paper Chromatography.

The major ether-soluble phytotoxins were separated using descending paper chromatography. Materials were spotted on Whatman No. 3MM paper and the chromatograms developed with isopropanol:14% NH_4OH (4:1, v/v). The chromatograms were cut into 1-inch strips and bioassayed. A single highly toxic area with an R_F value of 0.5-0.6 was repeatedly obtained. Field-decomposed barley residue was extracted and run in the isopropanol:NH_4OH solvent. The results were the same as those obtained with laboratory-decomposed barley residues. Laboratory-decomposed cotton, cowpea, and soybean residues also gave a single toxic area on chromatograms.

Other solvent systems were used in attempts to increase resolution. The substances in the toxic area of the chromatogram obtained in the isopropanol:NH_4OH solvent were eluted, concentrated, and chromatographed using the following solvents: _n_-butanol:acetic acid:water 8:2:2 (v/v); ethanol:NH_4OH:water 80:5:15 (v/v); acetone:urea:water 60:0.5:40 (v/v). In each case, only one area proved to be highly toxic. The area was most concentrated in the _n_ -butanol:acetic acid:water solvent where it had an R_F value of 0.82-0.95.

Paper chromatography was also used to follow the production of phytotoxins in the laboratory over a 3-month period. At weekly intervals, water extracts were made and bioassayed for phytotoxicity. They were then extracted with ether, concentrated, and chromatographed in the isopropanol:NH_4OH solvent. Only the one highly toxic area was present on the chromatogram. The presence or absence of this area coincided with the presence or absence of phytotoxicity of the original crude water extracts.

The ether-soluble phytotoxins were chromatographed in the isopropanol: NH_4OH solvent and the paper was sprayed with pH indicators bromthymol blue and bromcresol green. Three acidic spots appeared in the toxic area. These three spots were bioassayed separately and substances from each spot inhibited seed germination and/or root elongation. The materials in these three spots also were ninhydrin positive, giving a reddish-brown color, but only when the solvent system contained NH_3. Thus, when n-butanol:acetic acid:water was used, the ninhydrin-positive area was not evident, although an acidic reaction in the three areas could be demonstrated by means of pH indicators. These three areas, in addition, did not show any reaction with diazotized sulfanilic acid (DSA).

The clear-cut relationship between acidic spots and phytotoxicity indicated that organic acids were involved. Recently, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, syringic acid, protocatechuic acid, and ferulic acid were identified in toxic extracts from stubble mulch fields. These compounds were cochromatographed with the unknowns in the isopropanol:NH_4OH solvent. No similarities in R_F values were obtained. Furthermore, the known compounds gave positive reactions with DSA.

Of organic acids with R_F values similar to the unknown phytotoxins, four aromatic acids, benzoic acid (BA), phenylacetic acid (PA), 3-phenylpropionic (PP), and 4-phenylbutyric acid (PB), corresponded most closely with the phytotoxins when compared by cochromatography and the color reactions on chromatograms obtained with ferric cyanide and silver nitrate reagents. These acids gave positive ninhydrin reactions only when chromatographed in a solvent system containing NH_3. Similarities were also noted in short-wave ultra-violet absorption, and in a negative reaction with DSA. The pungent odor of PA was similar to that of the ether extract of the unknown. Finally, bioassays of chromatographed and eluted authentic samples of PA, PP, and PB at 50 ppm and of BA at 100 ppm showed inhibition of tobacco seedling root elongation similar to that caused by the unknown.

The paper chromatographic comparison and bioassay data strongly suggested that one or more of the aromatic acids mentioned above were present in the ether extracts from decomposing residue. Samples for gas chromatographic analysis were obtained by eluting substances from each of the three areas exhibiting phytotoxicity on paper chromatograms developed with isopropanol:NH_4OH. The materials were eluted with water and the eluate was extracted with ether at pH 2.0 and concentrated to a standard volume. Samples of each of the four aromatic acids were carried through the same procedure and known and unknown materials were sent to the Stanford Research Institute, Palo Alto, California, to be analyzed by gas chromatography. The column used was glass (5 ft x 1/4 inch) packed with 20% diethylene glycol adipate (DEGA) + 3% H_3PO_4 on a 60/80 Gas-Chrome Q substrate. The column was conditioned for 1 hr at 275-300° C. A Micro-Tek 220 dual flame detector at a temperature of 225° C was used. The column temperature was maintained at 190° C and the inlet temperature was 250° C. N_2 gas at 45 ml/min was used as a carrier. A comparison of retention times indicated that unknown area A contained primarily PA with a lesser amount of BA; area B contained PP with a small amount of PA; and area C contained BA, PA, PP, and PB, in small amounts. The appearance of single peaks when a sample of the appropriate authentic acid was added to the unknown further confirmed the identification.

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Analysis by gas chromatography was repeated in our laboratory with a sample obtained from barley residue that had decomposed in the field. This sample contained BA and PA. Cotton, soybean, and cowpea residues were obtained from plants grown in the greenhouse and decomposed in the laboratory for 3 weeks. Gas chromatogrphic analysis of their ether extracts indicated that BA and PA were present. Quantitative analysis indicated that the concentration of these aromatic acids present in the residue extracts was sufficient to account for the toxicity of these extracts.

The present work shows that when green barley residues are decomposed in soils with high moisture, two major water-soluble phytotoxic fractions are present. The main fraction, soluble in ether at pH 2.0, contains phytotoxins accounting for approximately 60% of the total toxicity of the original water extract. The remaining ether-insoluble fraction contains a high concentration of electrolytes. Efforts to identify phytotoxic components were directed primarily to the ether-soluble fraction. It was found to contain four aromatic acids in concentrations sufficient to account for the observed phytotoxicity. These structurally similar acids were benzoic acid, phenylacetic acid, 3-phenylpropionic (hydrocinnamic) acid, and 4-phenylbutyric acid. These compounds, with the exception of benzoic acid, have not heretofore been reported as products of crop residue decomposition, nor have their phytotoxic properties been previously demonstrated.

Since the parallel studies using extracts of green barley decomposing in the field also showed the presence of benzoic acid and phenylacetic acid, and since these compounds were also present as phytotoxic decomposition products of such diverse species as cotton, cowpea, and soybean, it would appear that they are of common occurrence in nature when environmental factors are favorable. The presence or absence of one or more of the compounds in an extract may be a result of the environment during decomposition or the length of time the material had been decomposing.

SUCCESSIONS OF MICRO-ORGANISMS INVOLVED IN DECOMPOSITION OF GREEN BARLEY AND PHYTOTOXIN PRODUCTION.

Experiments were designed to determine the microbiological successions, involved in the decomposition of green crop residues, which might be responsible for accumulation of concentrations of phytotoxins, particularly phenyl acids. Because of reports that phytotoxins accumulated more readily under conditions of high soil moisture the study was carried out in soil held at different water contents.

Incorporation of Green Crop Residues in Soil of Different Moisture Contents.

Green barley (Hordeum vulgare L.) stems (85% moisture content, MC) were cut into 1.5 inch pieces each with a node at one end. Two hundred pieces were buried three inches below the surface of an arable field soil (Berkeley Clay) which had been sieved through a 0.5 inch mesh into galvanized iron cylinders 18 inches in diameter standing in trays of water 2 inches deep. The cylinders were filled with the soil surfaces 17.5, 27.5, 37.5, 47.5 and 57.5 cm above the constant water levels in the trays. The barley stem samples were placed 10, 20, 30, 40 and 50 cm above the water level. During the experiment the soil temperatures at the 3 inch level varied between 16-18 C (maxima) and 12-14 C (minima). At the same time one-liter beakers were filled with chopped green barley, air dry field soil and tap water (100 g wet weight : 150 g : 300 ml) as described earlier. Seven, 14, 21, 26 and 32 days after incorporation, ten stem pieces were removed and the populations of micro-organisms in the adhering soil and in the tissue itself were assayed as follows. Soil adhering to the ten stem pieces was brushed off and 1 g samples weighed. One g samples of stem tissue made up of small clippings from the nodal ends of the ten pieces were ground for about 5 minutes with pestle and mortar. Both soil and plant samples were dispersed in 99 cc of sterile tap water in 125 cc conical flasks and shaken at 212 rev/min. for one hour. Successive 10 fold dilutions of the soil and tissue suspensions were made until a dilution of 10^{11} was achieved.

Determinations of Populations of Fungi.

One ml aliquots of dilutions 10^3, 10^4 and 10^5 were incorporated with approximately 10 ml warm (48° C) Martin's peptone, dextrose, rose bangal medium in petri dishes. These were held at 100% relative humidity at laboratory temperature, about 22° C. After 1 week populations of total fungi and a number of genera were recorded. It was possible to record populations of yeasts, Penicillium, Aspergillus, Geotrichum, Mucor, Trichoderma, Fusarium, Cephalosporium, Stemphyllium, Gliocladium, Cunninghamella, Helicocephalum and Arthobotrys.

Determinations of Populations of Aerobic Bacteria and Actinomycetes.

One ml aliquots of dilutions 10^6 and 10^7 were incorporated with extract agar (1000 g air dry soil was shaken with 1 litre tap water and left to settle overnight, the soil extract was decanted and autoclaved with 2% difco agar). Bacterial populations were recorded after one week's incubation at room temperature. The actinomycete population was recorded after 10 days' incubation at room temperature. A stereoscopic microscope was used to distinguish actinomycete from bacterial colonies.

Determinations of Populations of Anaerobic Bacteria.

Anaerobic bacterial populations were assayed by two methods:

Deep stab technique. Soil dilutions were made anaerobically. The dilution tubes contained 9 ml of a distilled water solution of 0.02% sodium thioglycolate and 0.0001% resazurin. These were boiled until colorless and autoclaved with rubber stoppers in place. Only tubes which remained colorless on cooling, indicating their essentially anaerobic condition, were used to prepare dilutions. The 1 g sample of soil or tissue, ground with a pestle and mortar under O_2 free N_2, was placed in a small open plastic vial. This was gassed with O_2 free nitrogen for 5 minutes and then dropped into the first dilution tube which was simultaneously gassed with O_2 free N_2. The dilution tube was agitated on a cyclo-mixer for 30 seconds and subsequent dilutions to 10^{15} were made by removing 1 ml samples with pregassed, sterile plastic disposable syringes with 3.5 cm long, 18 g needles and injecting into 9 ml aliquots of dilution medium. The same syringes were used to inoculate tubes of anaerobic medium with 1 ml of the corresponding dilutions. The anaerobic medium had the following composition. Beef extract 10 g, NaCl 5 g, K_2HPO_4 2 g, Difco proteosepeptone 10 g, dextrose 5 g, sodium thioglycollate 0.5 g, methylene blue 0.002 g or resazurin 0.001 g and Difco agar 1 g. The medium was made up to 1 liter with tap water and its pH after autoclaving was 6.5 Resazurin is a more sensitive E_o indicator than methyl blue and it was used in all but the first experiment.

Medium tubes were prepared anaerobically as described by Moore with 5 ml of a 1:1 parafin wax: petroleum jelly mixture layered on top of the tubes prior to autoclaving. When the tubes were inoculated the rubber stoppers were removed and while gassing with O_2 free N_2 the 1 ml dilution was injected through the parawax seal. A warm glass rod was then used to reseal the parawax and the rubber stoppers replaced. The inoculated tubes were incubated for 10 days at 22° C and the increase in turbidity, was measured using a Klett-Summerson colorimeter. A series of tubes were not inoculated and served as sterile controls. The endpoint dilution of growth was taken to indicate the population of anaerobic bacteria in the original sample.

Anaerobic dilution plate method. Dilutions were prepared as described above. The anaerobic medium was also similar to that described except it contained 2% Difco agar. This medium was prepared anaerobically and 40 ml aliquots poured into 500 cc conical flasks with two side arms. Prior to the medium transfer the flasks were gassed for 30 minutes with O_2 free N_2. The gas was introduced through a side arm level with the agar surface and exhausted from the side arm at the top of the flask which remained sealed with a rubber stopper. The flasks were sterilized with toppers wired in place and with clamped rubber tubes over the side arms.

When cool, the dilution tubes, flasks and sterile disposable syringes with 18 G 1.5 inch needles, were placed in an isolator made of a large polyethylene bag into which were taped two polyethylene gloves. The bag was gassed with O_2 free N_2 from 30 minutes prior to and throughout the manipulative procedure of making the dilutions. Two 1 ml aliquots of each dilution were injected into two flasks through the edge of the rubber bungs and distributed over the agar surface. These flasks were incubated at room temperature and after 10 days the numbers of colonies were counted. The plastic isolator is not shown for photographic convenience. In both procedures any dilution or media tube or flask which colored during use was rejected.

Organisms Isolated and their Relative Frequency.

Tables 1 and 2 give respectively a detailed analysis of the successions isolated from soil adhering to green barley straw and from barley straw itself.

The notable feature of these successions was the marked enrichment of anaerobic bacteria and the dominance of Geotrichum and yeast in the

Table I Populations of microorganisms/g soil adhering to barley stem pieces decomposing in soil with different moisture contents.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL					
		0	7	14	21	26	32
Laboratory conditions 100 g green barley 300 ml H_2O 150 g air dry soil	Aerob. Bact.	8×10^{8}	4×10^{9}	2×10^{9}	1×10^{8}	1×10^{7}	7×10^{6}
	Anaerob. Bact.	1×10^{5}	1×10^{8}	1×10^{9}	1×10^{10}	1×10^{12}	1×10^{10}
	Actinos.	5×10^{6}	3×10^{6}	1×10^{5}	2×10^{5}	1×10^{6}	6×10^{5}
	Fungi	1.5×10^{6}	2.4×10^{6}	5×10^{7}	2×10^{7}	2×10^{7}	3×10^{7}
	Geot.	0	1.2×10^{6}	3×10^{7}	2×10^{7}	2×10^{7}	2.9×10^{7}
	Yeast	0	1×10^{4}	2×10^{7}	2×10^{6}	7×10^{5}	3×10^{4}
	Pen.	1×10^{5}	1×10^{4}				
	Trich.	6×10^{4}					
	Asp.	6×10^{4}					
	C.P.	2×10^{4}					
	Fus.	4×10^{4}					

Table I cont.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL					
		0	7	14	21	26	32
Soil held 10 cm above water table M C - I9.4 %	Aerob. Bact.		1×10^8	3×10^8	6×10^7	4×10^6	8×10^6
	Anaerob. Bact.		1×10^7	1×10^8	1×10^7	1×10^8	1×10^7
	Actinos.		2×10^6	3×10^6	2×10^6	1.3×10^6	1×10^6
	Fungi		2.5×10^5	2×10^5	7×10^5	6×10^6	3×10^6
	Pen.		1.3×10^5	8×10^4	4×10^4	1×10^6	2×10^5
	Yeast		1×10^5	3×10^4	4.5×10^5	3×10^6	2×10^6
	Trich.		1×10^4				
	Fus.		1×10^4	2×10^4	1×10^4		
Soil held 20 cm above water table M C - I7.2 %	Aerob. Bact.		2×10^7	5×10^7	3×10^7	1×10^9	3×10^8
	Anaerob. Bact.		1×10^7	1×10^6	1×10^6	1×10^7	1×10^7
	Actinos.		2.3×10^6	2.5×10^6	4.7×10^6	2×10^6	3×10^6
	Fungi		3.7×10^5	1.5×10^5	2×10^5	5×10^5	6×10^5
	Pen.		2×10^5	8×10^4	2×10^5	2×10^5	3×10^5

Table I cont.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL					
		0	7	14	21	26	32
Soil held 20 cm above water table (continued)	Yeast		8×10^4	3×10^4		2×10^5	1×10^4
	Stem.		2×10^4				
	Fus.		2×10^4				
Soil held 30 cm above water table M C - 15.5 %	Aerob. Bact.		3×10^7	5×10^7	6×10^7	3×10^7	4×10^8
	Anaerob. Bact.		1×10^6	1×10^6	1×10^5	1×10^6	1×10^5
	Actinos.		3×10^6	1.6×10^6	3.1×10^6	8.5×10^5	1×10^6
	Fungi		2.1×10^5	8×10^5	2×10^5	2×10^5	1×10^6
	Pen.		1.2×10^5	1.5×10^5	1.3×10^5	8×10^4	4×10^5
	Yeast		1×10^4	4×10^4	4×10^4	5×10^4	1×10^4
	Mucor		1×10^4	1×10^4			
	Trich.		1×10^4	1×10^4			
	Fus.		1×10^4				
	Asp.				1×10^4		

Table I cont.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL					
		0	7	14	21	26	32
Soil held 40 cm above water table M C - II.4 %	Aerob. Bact.		2.4×10^7	1.7×10^7	8.4×10^6	3×10^7	9×10^6
	Anaerob. Bact.		1×10^5	1×10^7	1×10^6	1×10^6	1×10^5
	Actinos.		2.4×10^6	1.9×10^6	1×10^6	3.5×10^6	8×10^6
	Fungi		6×10^4	3×10^5	1.4×10^5	2.5×10^6	7.5×10^6
	Pen.		4×10^4	5×10^4	6×10^4	9×10^5	7×10^5
	Yeast		1×10^4	1×10^5	6×10^4	8×10^5	1×10^6
	Mucor		1×10^4				
	Fus.		1×10^4	3×10^4	1×10^4		
	Trich.				1×10^4		
Soil held 50 cm above water table M C - 8.3 %	Aerob. Bact.		5.4×10^6	3×10^6	2.8×10^6	1.1×10^8	7×10^7
	Anaerob. Bact.		1×10^5	1×10^6	1×10^6	1×10^5	1×10^5
	Actinos.		2×10^6	9×10^5	8.7×10^5	1.2×10^6	5×10^6
	Fungi		1×10^5	1.6×10^5	5.7×10^5	6×10^5	8×10^5

Table I cont.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL					
		0	7	14	21	26	32
Soil held 50 cm above water table (continued)	Pen.		6×10^4	7×10^4	1.4×10^5	1×10^4	2×10^5
	Yeast		1×10^4	4×10^4	3×10^4	5×10^5	5×10^5
	Mucor		1×10^4	5×10^4			
	Stem.		1×10^4		2×10^4	1×10^4	
	Fus.		1×10^4		1×10^4		

Aerob. Bact. = Aerobic Bacteria
Anaerob. Bact. = Anaerobic Bacteria
Actinos. = Actinomycetes
Geot. = Geotrichum
Pen. = Penicillium
Trich. = Trichoderma
Asp. = Aspergillus
C.P. = Conidial Phycomycetes
Fus. = Fusarium
Stem. = Stemphyllium

Table 2 Populations of microorganisms/g green barley stem tissue decomposing in soil with different moisture contents.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL				
		7	14	21	26	32
Laboratory conditions 100 g green barley 300 ml H_2O 150 g air dry soil	Aerob. Bact.	1.8×10^9	8×10^7	3×10^8	2×10^7	1.4×10^9
	Anaerob. Bact.	1×10^6	1×10^9	1×10^{10}	1×10^{13}	1×10^{12}
	Actinos.	1.3×10^6	8×10^4	1×10^5	1.7×10^6	2.3×10^5
	Fungi	6×10^5	3×10^7	2×10^7	9.6×10^6	7×10^7
	Yeast	2×10^5	1×10^7	1×10^7	0	5×10^6
	Geot.	1×10^5	2×10^7	1×10^7	9×10^6	6×10^7
Soil held 10 cm above water table, M C - 19.4 %	Aerob. Bact.	2×10^9	7×10^7	5×10^7	1×10^9	4×10^8
	Anaerob. Bact.	1×10^6	1×10^8	1×10^8	1×10^9	1×10^8
	Actinos.	1.1×10^5	1×10^6	8×10^5	4×10^5	5×10^5
	Fungi	9.4×10^6	3.8×10^6	1.7×10^7	2.8×10^6	4×10^6
	Yeast	8×10^6	3.6×10^6	1.4×10^7	2.6×10^6	3×10^6
	Geot.	1×10^4				

Table 2 cont.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL				
		7	14	21	26	32
Soil held 10 cm above water table (continued)	Mucor	1×10^4			1×10^5	
	Pen.	1×10^4	2×10^5		1×10^5	
Soil held 20 cm above water table	Aerob. Bact.	9×10^8	3.3×10^8	5×10^7	8×10^8	1×10^9
M C - I7.2 %	Anaerob. Bact.	1×10^6	1×10^7	1×10^7	1×10^8	1×10^8
	Actinos.	3.5×10^5	1.7×10^6	9×10^5	2×10^5	8×10^5
	Fungi	2×10^6	4.6×10^6	7×10^5	4×10^6	9×10^5
	Yeast	1.2×10^6	3.5×10^6	3×10^5	3×10^6	7×10^5
	Pen.	1×10^5	7×10^5		6×10^5	1×10^5
	Mucor	3×10^4				
	Fus.	1×10^4				
	Trich.			1×10^5		
Soil held 30 cm above water table	Aerob. Bact.	6×10^8	6×10^8	1×10^8	1×10^9	2×10^9
M C-I5.5 %	Anaerob. Bact.	1×10^6	1×10^6	1×10^7	1×10^9	1×10^6

Table 2 cont.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL				
		7	14	21	26	32
Soil held 30 cm above water table (continued)	Actinos.	3.5×10^5	3×10^6	9×10^5	1×10^4	2×10^6
	Fungi	5.2×10^6	5×10^5	3.2×10^5	6×10^5	1×10^6
	Yeast	5×10^6	5×10^5	1×10^5	5×10^4	5×10^5
	Pen.	2×10^5		2×10^5	4.5×10^5	3×10^5
	Mucor	1×10^4				
Soil held 40 cm above water table M C - II.4 %	Aerob. Bact.	2.2×10^8	4.5×10^8	6.6×10^7	6×10^8	4×10^9
	Anaerob. Bact.	1×10^6	1×10^7	1×10^7	1×10^6	1×10^6
	Actinos.	8×10^5	8×10^5	3×10^6	3.5×10^6	2×10^7
	Fungi	5.1×10^6	1×10^7	4.2×10^6	2.2×10^7	9×10^7
	Yeast	5×10^6	5×10^6	4×10^6	7×10^6	2×10^7
	Pen.	7×10^4	3×10^6	2×10^5	1.5×10^7	4×10^7
	Fus.		1×10^6			

Table 2 cont.

		DAYS AFTER INCORPORATION OF BARLEY INTO SOIL				
		7	14	21	26	32
Soil held 50 cm above water table M C - 8.3 %	Aerob. Bact.	1.6×10^8	1×10^7	5.6×10^7	7.4×10^8	1×10^8
	Anaerob. Bact.	1×10^5	1×10^6	1×10^6	1×10^6	1×10^5
	Actinos.	7×10^5	7×10^5	2×10^7	1.5×10^6	1×10^7
	Fungi	7.5×10^5	1×10^5	6×10^5	1.4×10^7	1×10^8
	Yeast	6×10^5		4×10^5	1.4×10^7	8×10^7
	Pen.	2×10^4	7×10^4	1×10^5		
	Fus.	1×10^4	3×10^4	1×10^5		1×10^5
	Mucor		3×10^4			1×10^5

Aerob. Bact. = Aerobic Bacteria
Anaerob. Bact. = Anaerobic Bacteria
Actinos. = Actinomycetes
Geot. = Geotrichum
Pen. = Penicillium
Trich. = Trichoderma
Fus. = Fusarium

laboratory beakers, while the successions in the cylinders of different moisture contents, under conditions closer to those anticipated in the field, were similar to each other and did not exhibit signs of anaerobic enrichment. The soil held in the cylinders 10, 20, 30, 40 and 50 cm above a constant water level had moisture contents ranging from 19.4% to 8.3%.

Bioassay for Phytotoxicity of Cold Water Extracts from Tissues with Known Microbial Successions and Analysis of Phenyl Acid Concentrations.

Barley stem pieces were held in soil columns at various moisture levels. At intervals they were removed, weighed and extracted with their own weight of cold water. This was done by allowing ice placed on top of the stems to melt over an 8 hour period so that the cold water trickled down through them. The wet mass of barley stems was squeezed in two layers of cheese cloth and the liquid obtained was centrifuged at 30.000 g at 0° C and filtered through a G.S. 0.22 millipore filter. The tissue decomposing in the beakers was extracted in the same way.

Small pieces of organic residue are the sites of phytotoxin production. The theoretical concentration of phytotoxins which comes into contact with a root tip growing in close proximity to a piece of residue will be that steady state concentration of soluble materials diffusing out of the residue into the soil solution. A root which penetrates residue pie pieces will be in contact with the actual concentration of phytotoxins in the production site. A cold water extract of the type used should contain a lower concentration of soluble materials than are actually present within the tissue from which the extract is made.

A bioassay of the phytotoxicity of the crude extract and various dilutions was performed as follows: A 0 cm seed germination paper in a petri dish was soaked with 5 ml of test solution and 20 lettuce seeds were placed on it. A wet 11 cm Whatman No. 1 filter paper was placed in the petri lid to maintain a high relative humidity. The dishes were incubated at 22° C at an angle of 10° from the vertical so that the roots grew downwards in response to gravity closely appressed to the germination paper. Lettuce seed will adhere to wet germination papers at the steep angles employed. After two days the percentage germination and lengths of the roots were recorded.

The phytotoxicity of the cold water extracts of green barley decomposing under different conditions of soil moisture content is shown in Table 3. The extracts of tissue decomposing under laboratory conditions were approximately 5 times more toxic throughout the period of the experiment than extracts made from tissue decomposing in the soil cylinders.

The symptoms of the phytotoxin injury ranged from a slight inhibition of seedling root elongation through a gradual foreshortening of the whole root system to a blackening of the root tip with the absence of any root hairs and finally to failure of the seed to germinate accompanied by blackening of the radicle end.

Gas chromatographic assay for phenyl acids in the cold water extracts. The volume of the cold water extract remaining after bioassay, measured and brought to pH 2.0 with IN hydrochloric acid was extracted 3 times with equal volumes of peroxide free diethyl ether. The ether extracted fractions were evaporated to near dryness at low temperature

Table 3. The percentage of inhibition of lettuce seedling roots by dilutions of cold water extracts of green barley residue decomposing under different conditions of soil moisture for different periods of time.

Soil conditions	*Dilution	Days after incorporation.				
		7	14	21	26	32
Lab conditions	I : 0	100**	100	100	100	100
	I : I	100	100	100	90	100
	I : 5	75	100	100	50	65
10 cm M C - I9.4 %	I : 0	50	100	100	100	100
	I : I	60	55	90	70	60
	I : 5	5	20	20	25	5
20 cm M C - I7. 2 %	I : 0	40	60	90	100	100
	I : I	40	30	60	35	45
	I : 5	10	15	20	15	5
30 cm M C - I5.5 %	I : 0	35	100	50	65	100
	I : I	25	20	35	60	60
	I : 5	10	15	10	20	25
40 cm M C - II. 4 %	I : 0	40	75	100	95	65
	I : I	30	45	50	45	30
	I : 5	5	10	15	15	10
50 cm M C - 8.3 %	I : 0	50	70	100	65	70
	I : I	30	45	55	30	40
	I : 5	10	10	25	5	15

* Ratio, crude extract : tap water

** Percentage inhibition of 2 day old lettuce seedling root growth compared with controls; average of two experiments.

and the oily residues dissolved in recorded volumes of 95% ethanol and stored at -20° C in sealed plastic vials.

These samples were analysed by gas chromatography using a Varian 1800 series gas chromatograph with a stainless steel column (5' x 1/4") packed with 20% diethylene glycol adipate (DEGA) - 3% H_3PO_4 on a 60/80 gas chrome Q substrate. A hydrogen flame ionization detector at 225 C was used. The hydrogen flow rate was maintained at 25 ml/minute. The column temperature was maintained at 190° C and the injector temperature was 250° C. Nitrogen was used as the carrier gas with a flow rate of 90 ml/minute.

Internal standards of benzoic, phenyl acetic, phenyl butyric and hydrocinnamic acids and comparison of retention times were used to identify and quantitize these four compounds in the sample extracts. In addition the presence of five other compounds and the relative concentrations of three of them were recorded.

The results of the gas chromatographic analysis are shown in Table 4. Higher concentrations of the four identified phenyl acids (particularly hydrocinnamic and phenyl butyric) and the five unidentified compounds were consistently found in extracts of decomposing residues carried out in beakers under saturated conditions.

Production of Phytotoxins by Organisms Isolated from Decomposing Green Residues.

One litre glass beakers were filled with 100 g wet weight green barley (87% M.C.) and 150 g air dry heat sterilized soil and the mixture

Table 4. Concentrations of four identified phenyl acids and relative concentrations of five unidentified compounds in cold water extracts of barley and rye decomposing for different periods of time under different conditions. (Extraction - I : I on a wet weight basis)

Conditions	Tissue	Period of decomp. in days	Benzoic* acid	Phenyl* acetic acid	Hydro* cinnamic acid	Phenyl* butyric acid	1**	2**	3**	4^a	5^a
Lab. cond.	Green barley	21	87	115	95	26	48	32	17	+	+
"	"	32	40	79	140	33	23	16	12	+	+
"	Green rye	21	35	80	210	35	56	25	20	+	+
10 cm above H_2O	Green barley	21	18	12	1.5	T	8	7	T	+	-
"	"	32	11	3.6	3.0	1	15	7	T	+	-
20 cm above H_2O	"	21	16	5	4	T	6	5	T	+	-
"	"	32	15	3.7	T	T	3	3	-	-	-
30 cm above H_2O	"	21	19	21	8	2	11	3	T	-	+
"	"	32	12	10	T	-	5	2	T	-	-
40 cm above H_2O	"	21	5	1.4	-	-	2	12	-	+	-
"	"	32	8	2	T	-	5	5	-	-	-
50 cm above H_2O	"	21	11	3.2	1.0	-	4	-	T	-	-
"	"	32	5	1.7	T	-	8	T	T	-	-

T - Trace, too small to measure. * - ppm measured gas chromatographically.

** - Relative concentrations in 0.1 mg weight of paper peak cut out of chromatogram.

[a] - Presence (+) or absence (-) of peak.

sterilized by a 48 hour exposure to propylene oxide. These flasks were then infested with 300 ml of sterilized tap water containing inoculum at a concentration of 10^6 cells/ml of organisms isolated from the previously studied microbial successions. These organisms were 2 isolates of yeasts, 2 isolates of *Geotrichum*, a *Clostridium* sp. and *Clostridium sporogenes*. The extraction and analytical procedures used were identical to those already described and were carried out after 21 days of decomposition.

Table 5 shows the concentrations of phenyl acids in 21 day decomposition extracts of green barley to which known organisms were added under saturated conditions determined by gas chromatography. The addition of any of the test organisms resulted in increased concentrations of benzoic and phenyl acetic acids in the decomposing residues. The two *Clostridium* spp. produced less of these acids than the yeasts or *Geotrichum* spp, but more hydrocinnamic and phenyl butyric acids. The concentrations of phenyl acids found after 21 days of degradation by only one organism were not as great as those found when the degradation was being effected by a range of microorganisms naturally selected from the soil.

Table 5. Concentrations of the four phenyl acids in ppm and percentage of five unidentified compounds in cold water extracts of green barley decomposed for 21 days by the named organisims in saturated conditions.

Organisms added	Benzoic* Acid	Phenyl* acetic acid	Hydro-* cinnamic acid	Phenyl* butyric acid	1^a	2^a	3^a	4^a	5^a
Yeast #I	21	25	-	-	-	-	-	-	-
Yeast #2	41	16	-	-	-	-	-	-	-
Geotrichum #I	28	12	+	-	-	-	-	-	-
Geotrichum #2	16	8	+	-	+	-	-	-	-
Clostridium sp.	8	6	11	8	-	+	-	-	-
Clostridium sporogenes	6	10	5.5	3.4	+	-	-	-	-
No organisms	2.6	1.9	-	-	+	-	-	-	-

* Concentration in ppm.

[a] Presence (+) or absence (-) of peak.

FIELD EVALUATION OF PHYTOTOXIN CONCENTRATION IN DECOMPOSING RESIDUE AND POTENTIAL SIGNIFICANCE.

The identification of some of the phytotoxic compounds produced during crop residue decomposition provided an opportunity to evaluate their occurrence under field conditions. This type of evaluation was necessary to determine their potential significance as a factor influencing the growth of crop plants.

Fields and Crops.

In early March of 1968, 26 fields in the Salinas Valley, California, were selected bearing cover or rotation crops of green barley, rye, vetch, broccoli and cauliflower. The soil types and crops are shown below.

Crops and Soil Types Used In Field Experiments.

Field No.	Crop.	Soil Type.
1	Vetch	Salinas silty clay loam
2	Barley	Salinas clay
3	Rye	Salinas fine sandy loam (overwash phase)
4	Rye	Salinas silty clay loam
5	Barley	Salinas silty clay loam
6	Rye	Montezuma clay
7	Rye	Chaular sandy loam
8	Vetch	Chaular sandy loam
9	Barley	Caular sandy loam

(continued next page)

Field No.	Crop.	Soil Type.
10	Barley	Hanford sand
11	Barley	Chaular loam
12	Rye	Hanford sand
13	Barley	Hanford sand
14	Rye	Salinas silty clay loam
15	Barley	Metzrey fine sandy loam
16	Rye	Salinas clay
17	Barley	Salinas clay
18	Cauliflower	Salinas clay
19	Broccoli	Salinas fine sandy loam (overwash phase)
20	Broccoli	Salinas silty loam
21	Barley	Salinas silty loam
22	Barley	Salinas silty loam
23	Broccoli	Salinas silty loam
24	Cauliflower	Salinas silty clay loam
25	Barley	Greenfield coarse sandy loam
26	Cauliflower	Salinas clay
27	Lettuce	Hanford sand
28	Lettuce	Hanford sand
29	Broccoli	Hanford sand

Sampling Procedure and Observations.

Every seventh day all the fields were visited and the date when the cover crop was incorporated into the fields noted. After that time, each week, representative samples of about 500 g fresh weight of organic residue were removed from the soil at between the 3 and 6 inch depths. A representative soil sample was also removed and all samples were immediately placed in an ice chest for transport to Berkeley.

In most fields growers planted lettuce at least three weeks after incorporation of organic residue. The lettuce seedlings emerging from these plantings were dissected carefully from the soil and their roots critically examined. Roots which penetrated or were appressed to organic matter were very carefully examined for sites of injury coincident with location of organic material. In addition seedlings growing in adjacent fields which had not been covercropped were examined for the incidence of root lesions.

The same day that the residue samples were collected they were extracted with cold water in the manner previously described. The extracts were centrifuged at 30.00 **g** for 20 minutes at 0 °C. The pH and electrical conductivity of the extract was then measured following filtration through a G.S. 0.22 u Millipore.

A bioassay as described above was performed twice with each extract and the inhibition of germination and root elongation were measured. The control levels of germination and root elongation were determined using a 1:1 cold water extract of the cooresponding residue-free soil.

The remaining volume of each extract, after acidification to pH 2.0 with N HCI, was extracted 3 times with an equal volume of diethyl ether and an analysis of the phenyl acid concentrations made gas chromatographically in the same manner as described previously. A bioassay was performed on a number of the aqueous solutions from which the ether extractions had been made.

The microbial successions in the field residues were determined by grinding 10 g samples of residue removed from the field with pestle and mortor and performing dilution analysis of populations of fungi, actinomycetes and aerobic bacteria as described previously.

The percentage moisture of each field soil, in which the residues were decomposing, was determined on each assay day by oven drying soil samples.

The lettuce seedling bioassay was performed twice on seed germination papers saturated with 5 ml of aqueous solutions of 10, 50, 100, 500 and 1000 ppm benzoic, phenyl acetic, hydrocinnamic or phenyl butyric acids. The percentage germination and root lengths were measured after two days. The control germination papers were saturated with sterile tap water. A similar bioassay was carried out with 5 ml of the same concentrations of the phenyl acids added to 25 g of air dry Salinas fine sandy loam substituted for the germination paper in petri dishes. This bioassay was repeated using mixtures of all four acids having concentrations of 25, 125 and 250 ppm of each acid in the mixture.

By the same procedures described above bioassays on germination paper and on one soil type (Salinas fine, sandy loam) were carried out using a balanced mineral solution containing $NaCI$, $CaCI_2$, $MgSO_4$ and Na_2SO_4 salts in equal proportions by weight diluted to give conductivities of the bioassay solutions of 0.5, 1.0, 2.0, 2.5, 5, 10 and 20 millimhos/ cm^2 at 25° C.

Efficiency of the Phenyl Acid Extraction Procedures.

It was desirable at this point to perform some experiments to determine the efficiency of the cold water and diethyl ether extraction procedures with regard to phenyl acids. Two methods were used to determine the efficiency of the cold water extractions from decomposing crop residues.

Extraction to end point. On 14 occasions samples of organic residue three times larger than were normally collected were removed from the field. About one-third of each larger sample was extracted in the standard way with an equal weight of melting ice over an 8 hour period and squeezed in two layers of cheese cloth. This extraction procedure was repeated four times using six of the residue samples and the extracts separately collected. After centrifugation, millipore filtering and extraction with diethyl ether, the concentrations of benzoic and phenyl acetic acids in the successive extraction volumes were determined gas chromatographically.

The concentrations of benzoic and phenyl acetic acids extracted by five successive cold water extractions are shown in Table 6. Between 60 and 70% of the total amount of benzoic acid extracted was removed by the first extraction. The concentrations of phenyl acetic acid were so low that this compound could be detected only in the first cold water extraction in all except one case.

Both the quantity and volume of the added phenyl acids which were taken up and retained by the residues, were then known and expressed as ppm by weight of the wet residue. The usual melting-ice extraction procedure was then performed and the concentration of phenyl acids in this extract determined.

Table 6. The concentrations in ppm of benzoic and phenyl acetic acids extracted by successive cold water extractions of crop residue samples decomposing in the field.

Crop	Days after incorporation	Extraction #1				Extraction #2		Extraction #3		Extraction #4		Extraction #5	
		BA		PAA		BA^e	PAA^e	BA^e	PAA^e	BA^e	PAA^e	BA^e	PAA^e
		a	b	c	d								
Barley	11	3.3	100	1.5	100	T	0	T	0	T	0	0	0
Rye	15	5.1	65	1.4	100	2.0	0	0.7	0	T	0	0	0
Broccoli	15	13.1	64	1.0	100	5.2	T	1.8	0	0.5	0	T	0
Cauliflower	5	61.7	67	2.6	74	22.5	0.8	6.1	T	1.1	0	T	0
Cauliflower	12	21.6	68	2.6	100	7.5	T	2.5	0	T	0	T	0
Vetch	4	24.3	61	T*	100	10.1	0	4.2	0	0.8	0	0	0

T - Trace

a - ppm benzoic acid

b - percentage of total benzoic acid extracted by the first extraction process

c - ppm phenyl acetic acid extracted by the first extraction process

d - percentage of total phenyl acetic acid extracted by the first extraction process

e - concentration in ppm

Efficiency of recovery of added acids.

By substracting that part of the concentration which was attributable to the constitutive phenyl acids in the tissue (i.e., the concentration determined by the standard methods employed with the first third of the sample; assuming very little of this is lost in the immersion procedure) it was possible to arrive at a figure for the concentration of phenyl acids in the extract derived from the phenyl acids added during the immersion procedure. The efficiency of the extraction was then expressed as the percentage of the amount of phenyl acids added which were extracted by the melting ice.

To determine the efficiency of the ether extractions, 200 ml aqueous solutions, containing 100 or 250 ppm benzoic, phenyl acetic, hydrocinnamic and phenyl butyric acids were prepared and extracted in the standard way three times with equal volumes of peroxide-free diethyl ether. The oily residue, after low temperature evaporation, was taken up in alcohol and analyzed gas chromatographically for the concentrations of the phenyl acids.

The percentages of the amounts of the four phenyl acids in aqueous solutions of various concentrations removed by three ether extractions averaged 91% for benzoic, 87% for phenyl acetic, 88% for hydrocinnamic and 85% for phenyl butyric acid (Table 7).

Seedling Damage in the Field and Phytotoxicity of Cold Water Extracts.

Damage to lettuce seedling roots in the field did not generally correspond to coincidence of residues and roots. No damage that could be attributable to residues was found in heavy cover crop situations. The

Table 7. The percentages of phenyl acids added to crop residues recovered by a single 1 : 1, 8 hour, cold water extraction.

Crop	Days after incorporation	Benzoic acid added		Phenyl acetic acid added	
		50 ppm	100 ppm	50 ppm	100 ppm
Barley	4	72*	63	67	67
Barley	11	81	59	51	58
Barley	18	68	64	40	63
Barley	25	58	61	61	63
Rye	15	73	68	43	70
Rye	22	69	57	60	50
Rye	29	78	63	66	62
Broccoli	1	80	65	64	61
Broccoli	8	53	57	44	57
Broccoli	15	73	62	52	58
Cauliflower	5	65	74	45	59
Cauliflower	12	72	75	57	65
Vetch	4	77	68	62	68
Vetch	11	70	64	54	56

*Percentage recovery.

damage found on lettuce seedling roots in the field, for the most part, differed from that produced by phytotoxins in the bioassay.

The phytotoxicity, expressed as percentage inhibition of root elongation over the control level, for the cold water extracts of residues of different crops at successive times after incorporation into the field is shown in Table 8. Of the 134 samples bioassayed twice, 90 reduced elongation by 90% or more. During the first 4 weeks of decomposition most cold water extracts of all residues were highly phytotoxic. As the decomposition proceeded the incidence of phytotoxic extracts decreased, more rapidly with extracts of barley, rye or vetch and less rapidly with broccoli or cauliflower.

Toxicity of Identified Phytotoxins in Filter Paper and Soil Bioassays.

The root lengths measured in the bioassays of phytotoxins carried out on germination paper were compared with lengths measured in two soil types. It was evident that bioassays performed on germination discs were of an order of magnitude more sensitive to phenyl acid inhibition than those performed on either soil type. On soil, concentrations of phenyl acids below about 250 ppm were actually stimulatory to lettuce seedling roots. Mixtures of the four phenyl acids were not synergistic in their phytotoxic effects, indeed, they were antagonistic.

Toxicity of Balanced Salt Solutions in Filter Paper and Soil Bioassays.

Similarly root lengths measured in bioassays of balanced salt solutions carried out on germination paper were compared with lengths measured in Salinas fine sandy loam. The lettuce seedlings are considerably

Table 8. The phytotoxicity of cold water extracts of residues decomposing in the field before and after the ether extractions were made, the decrease in phytotoxicity and the conductivity of the original extract.

Sample Number	Phytotoxicity Before Ether Extraction	Phytotoxicity After Ether Extraction	Decrease in Phytotoxicity	Conductivity Before Extraction Millimhos/cm^2
1	39*	49*	10*	6.7
2	0	22	22	6.8
3	0	0	0	17.5
4	27	30	3	8.1
5	30	42	12	7.6
6	34	65	31	4.6
7	0	0	0	12.8
8	28	63	35	3.8
9	35	56	19	3.5
10	34	41	7	7.0
11	28	39	11	5.2
12	56	70	14	5.0
13	43	45	2	6.8
14	0	15	15	8.0
15	16	25	9	6.2
16	0	0	0	12.2
17	0	11	11	6.0
18	41	52	9	4.8
19	21	33	12	3.4
20	0	23	23	4.3

* Phytotoxicity = root length expressed as a percentage of the control root length.

more sensitive to high ionic concentrations on paper than they are in soil. On germination paper seedlings begin to show inhibition by solutions with conductivities greater than 2 millimhos/cm^2 and are about 50% inhibited by a 4 millimhos/cm^2 solution. In field soil the first signs of inhibition are observed when solutions of about 4 millimhos/cm^2 are added and 50% inhibition is achieved with addition of an 8 millimhos/cm^2 solution.

Gas Chromatography of Field Samples.

The concentrations in parts/million (ppm) of the four identified phenyl acids in 104 sample-extracts are shown in Table 9 together with relative concentrations (the weights, in 0.1 mg, of peaks cut from the chromatographic paper) of five unidentified compounds designated 1, 2, 3, 4 and 5 respectively. The patterns of phenyl acids and other compounds extracted and recorded by this particular method were characteristic for each incorporated crop. In a number of cases a slight increase in phenyl acid concentrations can be seen over the first two weeks. Generally decomposition of the organic material is accompanied by decrease in the concentrations of phenyl acids as indicated by decrease in peak heights except for phenyl acetic acid which often appears later in the decomposition process. Of the 104 samples analyzed 67 contained measurable amounts of benzoic acid, 87 contained phenyl acetic, 6 contained hydrocinnamic acid and 2 contained phenyl butyric acid. Although 90 samples were highly phytotoxic in the germination paper bioassay only 12 of these contained sufficient concentrations of phenyl acids, measured gas chromatographically to account for any phytotoxicity. Actually, the

Table 9. Some characteristics of I:I cold water extracts of decomposing crop residues in the field.

I, field no; 2, days after incorporation of crop; 3, ppm benzoic acid; 4, expected toxicity of this concentration of benzoic acid in the bioassay on germination paper-expressed as root length as a percentage of the control; 5, ppm phenyl acetic acid; 6, expected toxicity of this phenyl acetic acid; 7, ppm hydrocinnamic acid; 8, ppm phenyl butyric acid; 9, weight of gas chromatogram peak of unidentified compound no. I; (0.1 mg); 10, weight of peak of compound no. 2; 11, presence of compound no. 3; 12, presence of compound no. 4; 13, presence of compcund no. 5; 14, actual toxicity of extract in bioassay expressed as root length as a percentage of the control.

I	2	3	4	5	6	7	8	9	I0	II	I2	I3	I4
Vetch													
I	4	24	80	T	+	-	T	25*	I8	-	-	-	97
I	II	4.9	+	3.I	+	-	-	43	-	-	-	-	0
I	I8	I.9	+	I.I	+	-	-	7	5	-	-	-	35
I	25				No sample								38
I	32	-	-	0.8	+	-	-	-	-	-	-	-	93
I	39	I.2	+	T	+	-	-	-	-	-	-	-	60
I	46	-	-	0.7	+	-	-	-	-	-	-	-	I8
Rye													
3	2	2.7	+	0.6	+	-	-	T	T	-	-	-	34
3	9	I.2	+	-	-	-	-	-	26	-	-	-	62
3	I6	-	-	I.4	+	-	-	T	-	-	-	-	0
3	23	5.4	+	2.3	+	-	-	-	-	-	-	-	I08
3	30	-			No sample								I00
3	37	-	-	I.3	+	-	-	-	-	-	-	-	90
Rye													
4	5	-	-	0.9	+	-	-	-	-	-	-	-	62
4	I2	I8	-	0.5	-	-	-	22	3I	-	-	-	34
4	I9	-	-		No sample								65
4	26	-	-	3.3	+	-	-	T	T	-	-	-	9
4	33	-	-	-	No sample								I8
4	46	-	-	I.2	+	-	-	-	-	-	-	-	59
4	47	-	-	-	No sample								I09
4	54	-	-	0.7	+	-	-	-	-	-	-	-	65
Rye													
7	2	I.I	+	6	+	-	-	-	-	-	-	-	77
7	9	5.9	+	I.5	+	-	-	22	I4	-	-	-	I5
7	I6	T	+	T	+	-	-	I0	I2	-	-	-	42
7	23	0.7	+	-	-	-	-	I0	8	-	-	-	0
7	30	T	+	T	+	-	-	T	T	-	-	-	4I
7	37	I.5	+	-	-	-	-	-	6	-	-	-	74
7	44	-	-	-	No sample								93
7	5I	-	-	I.3	+	-	-	-	T	-	-	-	73
7	56	-	-	0.8	+	-	-	-	-	-	-	-	96
7	63	-	-	0.5	+	-	-	-	-	T	-	-	84

Table 9. Some characteristics of I : I cold water extracts of decomposing crop residues in the field. Continued.

I	2	3	4	5	6	7	8	9	IO	II	I2	I3	I4
Vetch													
8	IO	0.8	+	0.5	+	-	-	5	3	-	-	-	56
8	I7	-	-	0.3	+	-	-	-	4	T	-	-	2I
8	2.4	-	-	0.5	+	-	-	-	-	T	-	-	23
Barley													
II	4	-	-	2.8	+	-	-	2	T	T	-	-	63
II	II	3.3	+	I.5	+	-	-	I5	I5	T	-	-	I9
II	I8	I.9	+	0.8	+	-	-	-	3	-	-	-	95
II	25	-	-	2.I	+	-	-	-	-	-	-	-	99
Rye													
I2	8	2.3	+	I.3	+	T	-	T	-	-	T	-	54
I2	I5	-	-	0.6	+	-	-	-	-	-	T	-	98
I2	22	-	-	-	No sample								I50
I2	29	2.6	+	3.0	+	-	-	-	2	T	-	-	53
Rye													
I4	7	4.I	+	0.9	+	-	-	-	T	-	T	-	30
I4	I4	2.6	+	2.I	+	-	-	-	T	-	-	-	0
I4	2I	-	-	0.9	+	-	-	8	T	-	-	-	20
I4	28	-	-	-	No sample								5
I4	35	-	-	2.3	+	-	-	7	T	-	-	-	20
I4	42	-	-	I.7	+	-	-	4	T	-	-	-	33
I4	49	-	-	-	No sample								90
I4	56	0.9	+	0.4	+	-	-	-	-	-	-	-	I6
I4	63	-	-	I.7	+	-	-	-	-	-	-	-	87
Rye													
I6	I5	5.I	+	I.4	+	-	-	2	I2	-	-	-	39
I6	22	I.5	+	-	-	-	-	-	-	-	-	-	28
I6	29	0.8	+	-	-	-	-	-	T	-	-	-	50
I6	36	-	-	-	No sample								2I
I6	43	-	-	3.7	+	-	-	-	-	-	-	-	0
I6	50	0.7	+	I.7	+	-	-	-	-	T	-	-	27
I6	57	0.6	+	I.4	+	-	-	-	-	-	-	-	3I
I6	64	-	-	-	No sample								25
I6	7I	-	-	-	No sample								49
I6	78	-	-	I.2	-	-	-	-	-	-	-	-	I9
I6	85	-	-	I.2	+	-	-	-	-	T	-	-	37

Table 9. Some characteristics of I : I cold water extracts of decomposing crop residue in the field. Continued.

I	2	3	4	5	6	7	8	9	I0	II	I2	I3	I4
Cauliflower													
I8	5	62	64	2.6	+	-	-	-	II2	-	-	-	0
I8	I2	22	82	2.6	+	-	-	48	42	-	T	-	27
I8	I9	4I	70	4.2	+	-	-	80	I0	-	T	-	0
I8	26	4.5	+	2.4	+	-	-	II	-	-	-	-	0
I8	33	-	-	0.4	+	-	-	22	-	-	-	T	0
I8	40	-	-	4.I	+	-	-	-	-	-	-	T	0
I8	47	I4	89	-	-	-	-	I3	-	-	-	-	0
I8	54	-	-	-	No sample								22
I8	6I	3.8	+	I.I	+	-	-	I4	T	-	-	-	0
I8	68	-	-	-	No sample								6I
I8	75	-	-	0.7	+	-	-	-	-	-	-	-	57
I8	82	-	-	4.3	+	-	-	7	-	-	-	-	46
I8	89	-	-	0.8	+	-	-	-	-	-	-	-	5I
Broccoli													
I9	3	5.7	+	T	+	-	-	I06	24	-	-	-	43
I9	I0	8.3	99	I.3	+	-	-	I24	22	T	-	-	6
I9	I7	T	+	T	+	T	-	6	T	-	-	-	23
I9	24	I.8	-	I.8	+	-	-	I4	-	-	-	-	32
I9	3I	5.4	+	3.2	+	-	-	8	-	T	-	-	20
I9	38	-	-	-	No sample								I8
I9	45	-	-	-	No sample								0
I9	52	5.2	+	I.6	+	-	-	I6	T	T	-	-	I8
I9	59	4.5	+	2.0	+	-	-	T	T	-	-	-	0
I9	66	-	-	0.3	+	-	-	T	-	T	-	-	36
I9	73	I.4	+	I.4	+	-	-	-	-	-	-	-	47
Broccoli													
20	I	I0	96	T	+	T	-	T	I5	-	T	-	28
20	8	6.3	-	-	-	T	T	II	T	T	-	T	I5
20	I5	I3	9I	I.0	+	T	-	T	T	-	-	T	7
20	22	T	+	6.5	+	-	-	-	-	-	-	-	0
20	29	T	+	2.3	+	-	-	T	-	-	-	-	37
20	36	0.7	+	-	-	-	-	-	-	-	-	-	5I
20	43	0.7	+	T	+	-	-	-	-	-	-	-	I7
20	50	6.2	+	I.2	+	-	-	6	-	-	-	-	20
Barley													
22	I5	6.3	+	I.4	+	.9	-	92	42	-	-	-	I7
22	22	0.9	+	0.7	+	-	-	-	-	-	-	-	35
22	29	-	-	I.4	+	-	-	-	-	-	-	-	93
22	36	-	-	-	No sample								0
22	43	-	-	2.5	-	-	-	-	-	-	-	-	89

Table 9. Some characteristics of I : I cold water extracts decomposing crop residue in the field. Continued.

I	2	3	4	5	6	7	8	9	I0	II	I2	I3	I4
Broccoli													
23	I0	5.6	+	-	-	-	-	86	-	-	-	-	8
23	I7	2.7	+	-	-	-	-	6	-	-	-	-	I6
23	24	-	-	-	-	-	-	6	-	-	-	-	23
23	3I	-	-	-	No sample								84
23	38	-	-	.65	+	-	-	-	-	-	-	-	69
Barley													
25	7	32	75	T	+	-	-	20	-	-	-	-	0
25	I4	-	+	T	+	-	-	-	-	-	-	-	55
25	2I	I.5	+	-	-	-	-	T	3	-	-	-	64
Cauliflower													
26	2	20	83	I.I	+	-	-	49	-	-	-	-	0
26	9	4.0	+	0.9	+	-	-	36	-	-	-	-	64
26	I6	I5	88	2.I	+	-	-	66	-	-	-	-	0
26	23	6.8	+	0.6	+	-	-	45	-	-	-	-	22
26	30	0.8	+	5.9	+	-	-	20	-	-	-	-	0
26	37	-	-	-	No sample								0
26	44	-	-	7.2	98	-	-	-	-	-	-	-	0
26	5I	2.6	+	-	-	-	-	-	4	-	-	-	29
26	58	I.5	+	I.2	+	-	-	I4	3	-	-	-	I5
26	65	0.6	+	-	-	-	-	I5	-	-	-	-	62
26	72	I.6	+		-	-	-	-	-	-	-	-	I02
Lettuce													
27	2	-	-	I.0	+	-	-	90	-	-	-	-	I9
27	9	I.I	+	-	-	-	-	5	-	-	-	-	I4
27	I6	-	-	-	No sample								I8
27	23	2.0	+	0.6	+	-	-	7	I6	-	-	-	4
27	30	6.4	+	2.3	+	-	-	I9	-	-	-	-	0

+ - concentration of phenyl acids stimulate lettuce roots in germination paper bioassay.
T - trace of peak; too small to measure.
* - weight of peak in 0.1 mg.

majority of samples, 84, contained stimulatory concentrations of phenyl acids based on a bioassay of known concentrations.

Bioassays of 20 aqueous extracts from which the ether extractions had been made revealed that the level of phytotoxicity was decreased from 0 - 35% (with an average of 12%) by the extraction process.

RELATION OF CROP RESIDUE DERIVED PHYTOTOXINS TO SOIL-BORNE DISEASES.

Many factors, operating in the soil, determine whether a plant will be attacked by a root pathogen and the severity of subsequent root destruction. Further, certain root rots may be attributed to several pathogens acting together, rather than to any single organism. Adverse environmental factors are significant in such a complex and can be primary initiating agents. Since phytotoxic substances, often formed during decomposition of plant residues in soil, can have a broad spectrum of injurious effects on roots and stems of plants, such compounds might be important in etiology of root disease.

Effect of Phytotoxic Substances on Root Rot of Bean.

A study was made to determine whether phytotoxic products, because of their adverse effects on plant cells, also could influence pathogenesis of *Fusarium solani* f. *phaseoli*, *Thielaviopsis basicola* and *Rhizoctonia solani*.

Greenhouse-grown Pinto bean seedlings were excised when 6 inches tall, surface-sterilized, trimmed to 3-in. stem sections, and placed in petri-dish moist chambers.

The inoculation and treatment procedures in all these tests were carried out as follows: distilled water drops of about 0.005 ml each, containing about 50 washed macroconidia of a virulent clone of *F. solani f. phaseoli* grown on potato-dextrose agar (PDA) slants were placed on 3 separate spots on each stem. To these drops were added drops of the same size containing the various phytotoxic extracts to be tested.

The extracts were obtained by cold water extraction of barley residue decomposing in the field. Distilled water with the fungus spores was used on 1 set of controls. The toxic extracts added to distilled water, without fungus spores, were used in the other set of controls to determine toxicity of extracts to bean stem tissues. Due to the drop technique, the final concentration of phytotoxic substances in the drops was half that used in lettuce bioassays. In general, visible damage to bean stem tissues produced by the toxic extracts without the fungus was often negligible.

Twelve stems were used for each treatment and the extent of lesion development was recorded after 7-day incubation at room temperature. Lesion severity was rated as none, slight, moderate, and severe, depending on lesion size and amount of necrotic tissue. Under similar experimental conditions, solutions of KNO_3 increased pathogenesis of *F. solani f. phaseoli*; whereas glucose delayed it. Thus, the effect of these substances was compared with the effect of toxic decomposition products.

The results show that extensive lesion development took place when fungus spores were placed on bean stems along with various toxic extracts. Very small lesions were produced on bean stems in controls containing fungus spores in distilled water. Similarly, only a few superficial discolored spots were observed on bean stems where drops of the toxic extracts had been placed, without fungus spores.

There were differences in rapidity of lesion formation and extent of necrosis noted between the various extracts obtained from the different plant residues. Since only a limited number of extracts was tested, however, the precise relationship between type of residue, decomposition period, and disease severity could not be determined. The experiments indicated, however, that pathogenicity of *Fusarium* to bean can be greatly increased by substances obtained from plant residues decomposing in the field. In general, such an effect was most pronounced with extracts obtained during early stages of decomposition and tended to be less with extracts prepared from crop residues decomposing in the field for longer periods of time.

Direct comparison between water and ether extracts of the same residue was not possible. Apparently, more than one compound was involved and only some of these compounds were ether-extractable.

A few additional experiments were carried out in which drops of various toxic extracts were placed on bean stems, allowed to stand for 10 hr, and washed off; then, conidia (suspended in water) were placed on the same spots. Results similar to those described above were obtained and lesions were much more severe on stem sections pretreated with toxic extracts. The lesions, however, developed more slowly and 10-day incubation was required to obtain lesions of the same size obtained after 7 days when toxic extracts and fungus spores were added simultaneously.

Stimulation of Pathogenesis May be Due to Increased Exudation of Nitrogenous Substances from Host Cells in the Presence of Plant Residue Decomposition Products.

Since nitrogen sources enhanced pathogenicity and since in the present experiments similar or more severe lesion development was obtained with extracts of decomposing plant residue, an investigation was made on the mode of action of these extracts. The ether extracts were of special interest, since amino acids and sugars are insoluble in ether when extractions are made at approximately pH 7. Accordingly, ether extracts of rye, barley, broccoli, and broad-bean residues, at the same concentrations as in previous tests, were placed on excised bean stems. Drops of distilled water were placed on excised bean stems. Drops of distilled water were placed on the controls. The drops were removed after 12 hr and stems washed twice with distilled water. Fresh drops of water then were placed over the treated spots and were allowed to stand for an additional 8 hr. These drops then were spotted on Whatman No. 1 paper and tested for ninhydrin positive substances. Positive results were obtained with all extracts tested, indicating that nitrogenous compounds were liberated from the tissues after exposure to toxic decomposition products. No ninhydrin positive substances were present in the controls. Similar tests were made to determine the presence of sugars. Tests showed that traces of sugars were liberated from bean stem tissues after exposure to toxic extracts, but results were variable and inconclusive.

Root Rots of Bean Caused by *F. Solani f. phaseoli*, *T. basicola* and *R. solani* Were Increased in Greenhouse Experiments by Pretreating Roots with Toxic Decomposition Products.

Surface-sterilized bean seeds (var. Small White) were planted in Vermiculite and the seedlings allowed to grow for 10 days. They then were removed and the Vermiculite carefully washed away from the roots. Plants selected for uniformity and healthy root systems were divided into

4 groups. Roots of one group were exposed 1 hr to water extracts obtained from various decomposing residues. The extracts selected for use in these experiments were highly phytotoxic in earlier studies. To minimize the injurious effect on roots, these extracts were diluted 1:1 with distilled water before use. The extracts used in greenhouse experiments were obtained from rye residues, decomposing in the field for 7 and 16 days; barley, broccoli, and broadbeans, decomposing for 14, 16, and 10 days, respectively; and timothy, decomposing for 15 days. After exposure to the various toxic extracts or water controls, plants roots were placed for 10 min. in a spore suspension of about 1,000 spores/ml of F. solani f. phaseoli or T. basicola. In the case of R. solani, inoculum was obtained by removing and macerating the surface growth on 2 PDA slants. The second group of plants also was exposed to the extracts, but was not inoculated. The third group was placed in distilled water for 1 hr, instead of in the extracts; and then inoculated. The fourth group of plants was placed in distilled water for 1 hr and not inoculated. All groups then were replanted in Vermiculite, watered at 3 intervals with Hoagland's solution at half strength, and allowed to grow 21 days in the greenhouse. Then, plants were carefully removed from the pots, washed, and examined for root disease severity. Six plants were tested/treatment and each experiment was repeated 3-4 times.

When water extracts of timothy were used, no evidence of injury or of disease was visible in the above-ground portions of the plants during the course of the experiment; but marked differences in the severity of root damage were evident when the plants were examined at the end of the 3-week period. Lesions caused by *Fusarium* and by *Thielaviopsis* [illegible] on the roots of all plants inoculated with these organisms. The root-rot severity was greatly increased, however, where plant roots had been exposed to the extract prior to inoculation with the fungi. *R. solani* produced a few lesions on bean roots, but abundant invasion by the fungus was observed when roots were exposed to extracts prior to inoculation. Some damage was observed on roots treated with the extract alone, as evidenced by rootlet injury and discoloration of some main roots. The plants recovered rapidly, however, in the absence of the pathogens; and new healthy roots were formed after plants were repotted. The roots of control plants, receiving no treatment, were healthy.

The experiment was repeated with the same pathogens and with water extracts of rye residue decomposing for 7 days and of barley residue decomposing for 14 days, used separately and in addition to the timothy residue extract used previously. The results were similar to those obtained in the previous test; root-rot severity was greatly increased in all cases where roots had been exposed to the extracts prior to inoculation. Lesions caused by *Fusarium* and by *Thielaviopsis* were particularly numerous and severe on roots pretreated with the extracts. As a consequence, much of the root system of these plants was destroyed. *Rhizoctonia* again penetrated more readily those roots previously exposed to the extracts; whereas 22 of 36 segments yielded the fungus from roots pretreated with the extract. Some damage, noted in the previous experiment, was present on noninoculated roots exposed to the water extracts. Again, there was rapid recovery in the absence of pathogens.

Experiments using water extracts of broccoli decomposing for 10 days and of broadbean decomposing for 10 days, together with the timothy extract used previously, showed once again that exposure of roots to extracts greatly increased root rot of bean caused by *F. solani f. phaseoli*, *T. basicola*, and *R. solani*.

Effect of Phenyl Acid Phytotoxins and Phytotoxic Extracts on Root Rot of Cotton.

A study was also made to determine the involvement of phytotoxins in increasing *Thielaviopsis* root rot on cotton. Clones of *T. basicola* used in these studies were isolated from tobacco (*Nicotiana tabacum* L.) (T), cotton (*Gossypium hirsutum* L.) (C), and cherry (*Prunus* sp.) (CH). All clones were maintained as single conidium cultures on potato-dextrose agar (PDA) slants. Pure preparations of chlamydospores were prepared from these slants. These preparations were added to cotton field soil. After the chlamydospore chains had broken up, the soil was air-dried for several months. These chlamydospore-amended soils were then used in germination and inoculation studies on cotton seedlings (Acala 4-42).

Stimulation of chlamydospore germination by known phytotoxins or by phytotoxic extracts was determined by adding the test solutions (250 ppm) to small amounts of previously wet chlamydospore-amended (clines C and T) soil in porcelain cavity plates. Germination counts were made 20 hr later in soil smears. Distilled water and a 10% (by volume) solution of carrot juice were used as controls.

The predisposing capacity of the phytotoxins or of phytotoxic extracts of decomposing residues was determined on cotton seedlings by treating the seedling tap roots with the solutions prior to inoculation. Seedlings were grown in the greenhouse at a constant 32 C for 3-4 days in U.C. Mix. They were removed from the mix before lateral roots appeared. The seedlings were gently washed to remove loose soil particles, immersed in the test solutions for 2 hr, washed again, and transplanted into hinged plastic inoculation chambers. The seedling tap roots were inoculated in two places with small units of chlamydospore-amended field soil that had been kept wet for several days prior to use. Water was used as a control treatment, and noninoculated treated seedlings were used to check toxicity of the test solutions. Toxicity was indicated by discoloration or decreased turgidity (as indicated by flexibility when sliced with a scalpel) of the root tissue. Known solutions were adjusted to concentrations where no toxicity was apparent. Concentrations of phytotoxins in unknown extracts were diluted to 1/50 the concentration of the original ether extract and, as with the known solutions, only used once. Clones C (virulent) and CH (nearly avirulent) were used in these studies. Clone CH was used to determine if its virulence could be increased by treatment of the host tissue with phytotoxins. Disease severity ratings were made at intervals up to 4 days, after which differences in lesion severity between treated and untreated seedlings were less obvious, especially when virulent clone C was used. Severity ratings (0-10 scale) were subjectively based on lesion size and degree of discoloration, numbers of chlamydospores whose germ tubes had penetrated host cells, and the degree of hyphal proliferation on the roots. The last two criteria were not used in all trials, but when used were based on microscopic examination of longitudinal hand sections of inoculated root tissue that had been washed thoroughly in a vigorous stream of water and stained with 0.02% acid fuchsin in lactic acid. These sections were made 24-48 hr after inoculation.

The possibility that toxins might directly stimulate germination of chlamydospores in soil was first investigated. Tests were made with wet chlamydospore-amended field soil (clones C or T) to which an ether extract of decomposing barley or the known phytotoxins at a concentration of 250 ppm were separately added. The results, based on four experiments wherein 100 chlamydospores per clone per treatment were counted, showed that chlamydospore germination did not occur in the presence of the phytotoxic barley ether extract or any of the four phytotoxic acids. Thirty to 65% germination (clones C and T, respectively) occurred, however, in carrot juice controls. No germination occurred in the water controls.

The possible predisposing activity of the phytotoxic acids to *T. basicola* root rot was therefore investigated. Cotton seedling roots treated with test solutions were inoculated with the strongly pathogenic clone C or the weakly pathogenic clone CH by placing small units of chlamydospore-amended soil, kept moist for several days prior to use, on the roots for 4 days, after which the resulting lesions were rated for disease severity. The averaged disease severity ratings from several experiments indicate that the barley ether extract, phenylpropionic acid, and to a lesser extent benzoic acid, increased the severity of root rot caused by the cirulent clone C. The effects of the barley ether extract and phenylpropionic acid were similar but less striking with the nearly avirulent clone CH. No phytotoxicity was evident at the highest concentrations of the compounds used. Clone C was used to determine the reason for the increase in disease on toxin-treated roots. Longitudinal hand sections of inoculated root tissue were made 24-48 hr after inoculation, stained, and examined microscopically. Chlamydospores which had germinated on the root surface, and whose germ tubes had penetrated the host, were much more numerous on toxin-treated roots than on the water-treated controls. The resulting lesions 67 hr after inoculation were more severe on the root treated with phenylpropionic acid than the water-treated root. Following penetration, the rate of hyphal proliferation, both on the root surface and within the host tissue, was much greater in toxin-treated than in the water-treated roots.

Breakdown of Root Rot Resistance in Cotton By Hydrocinnamic Acid.

Hydrocinnamic acid (3-phenylpropionic acid) was identified as one of the compounds formed during the decomposition of crop residues in soil. Studies were conducted to determine whether resistance in cotton to *Thielaviopsis basicola* root rot could be overcome by treating roots with this compound prior to inoculation.

Varieties of cotton (Gossypium arboreum L.), known to be susceptible (A-32) and resistant (A-25 and A-26) to *T. basicola* were grown for 3 days in U.C. mix (1) at 27 C in the greenhouse. These seedlings were removed and the tap roots treated for 2 hr in water or a 75-ppm solution of hydrocinnamic acid. The treated and control plants were then transplanted into plastic inoculation chambers, and the tap roots were inoculated at two loci with small units of field soil infested with chlamydospores of *T. basicola* (clone C isolated from cotton). This soil had been wet for several days. After 5 days at room temperature (approximately 23 C), the inoculation sites were rated for disease severity based on the degree of tissue discoloration and the extent of fungus proliferation. Ratings of 0 for no infection to 10 for maximum infection were used. The results of these experiments show that hydrocinnamic acid treatment of resistant roots rendered them susceptible to attach by *T. basicola*.

Our observations on the resistant varieties A-25 and A-26 confirm those made by others that chlamydospores of *T. basicola* do germinate to a limited extent on the roots of resistant or tolerant varieties, and epidermis penetration by their germ tubes does occur. However, subsequent proliferation of the fungus is restricted. Proliferation of the fungus on varieties A-25 and A-26 treated with hydrocinnamic acid, on the other hand, was not restricted. In addition, a greater number of chlamydospores were observed to germinate and give rise to infection sites.

The significance of this study lies in the fact that hydrocinnamic acid, a compound occurring naturally during the breakdown of crop residues in soil, can bring about a breakdown of T. basicola root rot resistance. In addition to having potential field significance, this phenomenon may yield insight as to the nature of the resistance mechanism in this and other host plants. Treatment of resistant roots with hydrocinnamic acid somehow increases the responsiveness of the fungus to stimulatory root exudates. In addition, when penetration did occur, the fungus was less restricted in a hypersensitive reaction. It is possible, then, that resistance of roots to attack by T. basicola involves some prepenetration mechanism as well as a post-penetration hypersensitive reaction. Hydrocinnamic acid may interfere with these mechanisms:
(i) by removing or preventing formation of normally inhibitory substances which might be emitted from and present in resistant variety tissues;
(ii) by increasing the exudation of stimulatory materials; or (iii) both.

THE EFFECT OF GREEN CROP RESIDUE DECOMPOSITION ON PYTHIUM ULTIMUM POPULATIONS AND INOCULUM POTENTIALS AND DAMAGE TO LETTUCE SEEDLINGS.

A number of pathogenic agents are known to be capable of causing damage to lettuce seedling roots. These include Rhizoctonia solani Kuehn Sclerotinia spp, Pythium spp, Pythium ultimum Trow., Botrytis cinerea Pers. ex Fr., high salt concentrations excessive fertilizer applications, anaerobiosis, nematode activity and excessively high temperatures. The seedling disorders of lettuce in California have not been considered to be very important in the past because of the high sowing rates which were commonly used.

Implication of Pythium ultimum.

In the two cases of field damage and stand loss examined in detail the following observations were made.

1.) The symptoms were characterized by severe damage to the main root tip (browning and disintegration of this tissue) damage to tips of emerging lateral roots and scattered discrete lesions on both hypocotyls and cotyledons.

2.) Pythium sporgania and oospores (P. ultimum in all but two cases where an identification was possible) were observed in 92% of the damaged root tips examined microscopically and Rhizoctonia solani mycelium was observed on 17% of the damaged hypocotyls.

3.) Pythium ultimum was isolated from 94% of the damaged root tips and all of the 20 clones were pathogenic towards lettuce seedlings in a test carried out in infested field soil. Damage was completely controlled by 100 ppm active Dexon (p (p (Dimethylamino) benzenediazo sodium sulfonate) supplied by Chemagro Corp.) applied as a suspension of 70% active wettable powder at seeding time to field soil in which 80% damage was observed in the controls.

It appears that *Pythium ultimum* is involved in the lettuce seedling damage examined in the summer of 1968. The purpose of this study was to determine the extent of *P. ultimum* damage to stands of lettuce and the possible effects of green cover crops on *P. ultimum* populations and inoculum potentials as defined by Garrett.

Plot Preparation, Sampling Procedures and Field Conditions.

1. *Preparation of plots*. In March of 1968 seven fields bearing heavy cover or rotation crops in the Salinas Valley were selected. Table 10 shows the crop-type yield/acre, and percentage moisture in the crop at time of incorporation, dates when crops were incorporated, incorporation procedure used by the grower (discing or roto-tilling), and total nitrogen in ppm in the soils prior to incorporation (measured by Roland Hutchings analytical laboratory, Salinas) and soil types.

Immediately after crop incorporation and before water was applied, two replicates of three plots were prepared in each field as follows. Soil was removed from an area 3 x 2 feet to a depth of 1 foot and sieved through a tilted 0.5 inch mesh screen. In this way one plot, designated 0, with the bulk of the residue removed was prepared. The residue removed from plot is now added to an adjacent 3 x 2 feet area and after sieving the soil a plot with twice the regular amount of reside was prepared, designated plot II. Finally a second adjacent 3 x 2 feet area

Table 10. Field number, type, yield / acre and percentage moisture of crop incorporated, date and method of incorporation, soil type and total nitrogen in fields where residue was incorporated.

Field number	Crop incorporated	Yield(1000 lbs/acre)	% moist. in crop	Date of inc.	Method of inc.	Soil type	Total N_2 ppm
1	Barley	17	87	3/25	Disc.	Salinas fine sandy loam, heavy phase.	2630
2	Barley	17	87	3/26	Disc.	"	2210
3	Rye	35	76	4/22	Disc.	Salinas silty clay loam.	2570
4	Broccoli	14	85	4/28	Roto tiller	"	2260
5	Rye	30	58	4/28	Roto tiller	"	2220
6	Barley	30	62	5/6	Disc.	Salinas silty clay loam	2350
7	Barley	14	82	8/30	Disc.	Salinas fine sandy loam, heavy phase.	2380

was sieved and the residue replaced together with the sieved soil to give a plot with the regular field concentration, designated plot I.

2. Sampling procedure for the first week. The same day that these plots were prepared representative soil samples were taken from each plot and residue samples removed from the field adjacent to the plots. Two hundred lettuce seeds (Great Lakes 659) were planted in 6 inch^2 area at exactly 0.25 inch depth and 100 live turnip seed "bait" were laid in two 1 foot lines across each plot, also 1.25 inches deep, on either side of the lettuce seed. The plots were then watered with 2 gal/plot applied from a watering can so that the top 6 inches of soil was saturated and an unglazed clay dish eight inches in diameter was placed over the lettuce seeded area. The function of the dish was to retard water loss so that sufficient moisture would be retained to allow lettuce seed germination and emergence of the seedlings. On the following day the turnip seed bait were removed and together with the soil and residue samples were returned to Berkeley where the tests described below were carried out. Each plot was watered with a further 1.33 gal.

3. Sampling procedure for the second week. Exactly one week after plot preparation the same plots were revisited. The 200 lettuce seedlings planted the week before were removed together with the soil in which they were growing. Soil and residue samples were also removed and the lettuce seed planting, turnip seed baiting and watering processes repeated. On the following day the baits were removed, the plots watered with 1.33 gal/plot, and all the samples returned to Berkeley, where in addition to the tests carried out the previous week an assay of lettuce

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seedling damage was made.

4. Soil temperature and moisture. It was intended that continuous recording of soil temperature would be made in some of the plots. However, suitable equipment was not available at this time so the daily maximum and minimum air temperatures recorded by the U.S.D.A. Sugar Beet Research laboratory in the Salinas Valley were used to give a rough indication of the trend in soil temperatures.

By carrying out a standard irrigation cycle of two days saturation, followed by slow drying on the subsequent five days, it was hoped that soil moisture as a variable affecting the residue incorporation would be standardized and hence could be ignored for this experiment.

Air temperatures increased over the period of the experiment. At the beginning of the experiment minima were around 10° C and maxima around 21° C while at the end of the experiment minima were around 16° C and maxima around 30° C.

Influence of Crop Residues on *P. ultimum* Populations.

The population of propagules of *P. ultimum* was determined in the soil samples using the excellent method developed by Stanghellini. Twenty g samples of soil were shaken with 80 ml sterile tap water in 125 cc conical flasks on a rotary shaker at 212 rev/min. for 1 hour. Ten ml of the resulting slurry were added to 90 ml of sterile tap water and shaken for a further five minutes. Drops of this 1:50 soil water dilution were spotted on to thin 2% water agar plates in unscratched plastic petri dishes and dried rapidly with a fan before the lids were replaced.

After 18 and 30 hours incubation in the light at laboratory temperature (22°C), the number of characteristic hyphal patterns of P. ultimum growing from each drop was recorded. The plates were held against a black background while illuminated from the side with fluorescent light for rapid identification of the hyphal patterns. The efficiency of this method in determining populations of P. ultimum/g of soil and the role of sporangia as survival structures has been discussed by Stanghellini. The results presented here are in agreement with his work. Sporangia were the origin of most developing P. ultimum colonies and single hyphal strands could be traced back to single propagules in 93% of the cases examined.

Of the 120 isolates identified 96% were called P. ultimum. These include isolates which from only the sporangial stage and isolates which produce sporangia enclosed in bulbous, antheridial type hyphae as well as normal sporangia and sexual stages.

P. ultimum populations ranged from a low of 42 to a high of 7460 propagules/g soil with a mean of 965 and a standard deviation of 1265. An incorporation of green residue consistently resulted in increased populations of P. ultimum above that in the corresponding control situation. The order of magnitude of this change was frequently two times and up to six times and the greater increases were generally observed where greater amounts of residue were incorporated. In all the plots, with or without the addition of residue, an as yet unexplained marked increase in P. ultimum populations began to occur at the beginning of June, peaked in the middle of June and was still evident in the middle of July. During this period populations increased from a few 100 to a few 1000 propagules/g reaching a maximum in one plot of 7460 propagules/g.

P. ultimum Populations and Damage to Lettuce Seedlings.

The populations of *P. ultimum* in the soil, in which the lettuce seedlings were growing, were determined. The soil, containing the lettuce seedlings, was then washed through a number 10 sieve, with 0.078 inch holes, and the percentage seeds showing damage to the tip of the main root determined. The seedlings were cleared in a freshly prepared solution of 12% ethanol and 15% sodium hydroxide at 60° C for 2 hours. After washing in three changes of water and staining in cotton blue several seedlings from each sample were examined for the presence of recognizable pathogenic fungi growing in the damaged tissue.

The damage occurring on the roots was identical to that previously observed. There were brown lesions on the root tips, particularly the main root tips, with disintegration of the tissue and discrete lesions on the cotyledons and hypocotyls.

For the first two or three weeks following green residue incorporation the damage to seedlings in the field was generally more severe in plots where the *P. ultimum* population was higher as the result of residue incorporation. In the following weeks the damage in plots in which residue had been incorporated, with higher *P. ultimum* populations, was in many cases lower than the damage in plots without residue. The damage in all plots increased over the period of the experiment and the increase was more marked in the plots to which residue had not been added.

A total of 350 selected seedling roots were examined microscopically. Pythium spp were observed in damaged tissue of 294 seedlings (84%), Rhizoctonia was seen on 56 (16%). Of the Pythium observations in which it was possible to identify a species 94% were P. ultimum, 3% P. splendens Braun and the remaining 3% an unidentified spiny oospored species.

Inoculum Potential of P. ultimum Measured by Turnip Seed Baiting.

The inoculum potentials of P. ultimum populations in the field towards germinating turnip seed, represented by percentage seeds colonized by the fungus, were determined. Plates for the identification of the Pythium species were prepared with wedges of rolled oat agar poured in glass petri dishes held at a 45° angle. Rolled oats agar was prepared by boiling 50 g rolled oats in 100 ml tap water for 10 minutes, straining through cheese cloth and incorporating the filtrate in 1/1 of 2% water agar. The dishes were returned to the horizontal position and 10 ml sterile tap water poured into them to form a thin layer of water in the bottom. These dishes are now termed rolled oats agar/ water plates. Each wedge was inoculated with a clone of Pythium isolated from damaged lettuce root tips or from soil by Stanghellini's method. After 7 days the mycelial mat growing over the water surface was examined microscopically and the species determined. A total of 120 clones were examined in this way.

This method was used only for the first few weeks but of the 31 field situations where it was used a definite trend was established. Where residue was incorporated and populations of P. ultimum increased the percentage turnip seed colonized also increased. Of the 31 sets of three plots (0, I and II) examined, in 24 cases the highest inoculum potentials, expressed as percentage seed colonized, were found in the plots where residue had been incorporated increasing *P. ultimum* populations. In 19 cases the lowest inoculum potentials were found in the plots which had received no residue.

Phytotoxicity of Water Extracts of Residue.

The phytotoxicity of water extracts of the residues was determined in bioassay on germination paper as described previously. Gas chromatographic analysis of a random selection of 10 samples extracted with diethyl ether was performed.

Most of the aqueous extracts were phytotoxic in the bioassay. The symptoms of phytotoxicity were the same as were observed in 1968. These may best be described, in their more severe form, as a darkening and collapse of the intact root tip and stunting of roots whilst lesions are not evident on cotyledons or hypocotyl. The concentrations of phenyl acids were also very similar to those obtained in 1968. Only benzoic and phenyl acetic acids were found with maximum concentrations of 23 ppm (B.A.) and 11 ppm (P.A.A.).

Effect of Residue Incorporation on Populations of P. ultimum, Other Organisms and Concentration of Soluble Nutrients in the Soil Solution.

Incorporation of residue and determination of micro-organism populations. Sixteen g fresh weight samples of broccoli, and young and old barley were incorporated into pots containing 1500 cc Salinas fine sandy loam. The broccoli had a moisture content of 76%, the young barley 83%, the mature barley 74% and the soil 22.1%. The methods of incorporation and watering were identical with those used in experiments 1 and 2. The soil into which these incorporations were made had been removed from the field three weeks previously and had then been stored in 8 inch pots in the greenhouse and watered at the surface every day. This was done so that a release of soluble nutrients, such as occurs when a dry soil is watered, would not occur and obscure the release of soluble nutrients from organic residues. Four replicates of each treatment were prepared, 16 pots in all, and they were placed in the greenhouse room with the constant diurnal temperature fluctuation between 19°C and 25°C. This incorporation procedure and preparation of pots was exactly repeated three weeks later using broccoli with a moisture content of 74%, green barley 85%, and mature barley 72%. Only the nutrients released into soil, not the populations of soil organisms, were measured in this duplicate experiment.

Twenty g composite soil samples, 5 g from each replicate of each treatment were removed 2, 8, 14 and 22 days after residue incorporation. The populations of P. ultimum, other fungi, actinomycetes and bacteria/g soil were determined by the previously described methods.

The results were similar to those previously obtained. All three organic residues when added to soil caused an increase in bacteria, actinomycetes and fungi, including P. ultimum. The green immature residues were more effective than the mature barley in bringing about this increase. The P. ultimum population increase appeared to have peaked at about 14 days and was decreasing 22 days after residue incorporation. Actinomycetes, on the other hand, increased their population throughout the 22 day experiment.

Soluble nutrients released into the soil solution. Four hundred g composite soil samples, 100 g from each replicate of each treatment, were removed 2, 8, 14 and 22 days after residue incorporation. Each sample was shaken vigorously by hand with 200 ml distilled water at 0°C for 100 shakes in about 30 seconds. The resulting slurry was immediately centrifuges at 0°C at 3.000 g for 20 minutes and the supernatant was then filtered through a G.S. 0.22 u Millipore. It was intended by this extraction procedure to remove primarily those soluble materials present in the soil solution and immedistely available to micro-organisms, and leave behind nutrients associated with organic particles and micro-organisms. This organism free, rapid cold water extract of soil, was divided into three fractions, two of which were subject to analyses for soluble carbohydrate or ninhydrin positive compounds.

The soluble carbohydrate concentrations were determined by the anthrone method using Bausch and Lomb Spectronic 20 spectrophotometer at 620 mu and a standard curve prepared with glucose to determine actual concentrations. Equal amounts (by weight) of different sugars react with anthrone (10- dihydro- 9-ketoanthracene) to give approximately equal color reactions and readings on a spectrophotometer.

The soluble ninhydrin positive compounds were determined by the ninhydrin method using the Spectronic 20 at 570 mu and a standard glycine curve to determine actual concentrations. Ninhydrin (tri-keto-hydrindenehydrate) reacts with ammonium salts, amino acids and other compounds. Equal yields of colored reagent have been shown to be produced by equimolar amounts of most common amino acids. This whole experimental procedure was repeated three weeks later in a duplicate experiment.

The green residue incorporation caused a three fold increase in the sugar concentration which peaks after 14 days. After 22 days the concentration had fallen to the level seen in the control soil. The mature barley incorporation caused less increase in sugar concentration which peaked after about 8 days and returned to the control level about 22 days after residue incorporation.

A very marked increase was observed in the concentration of immediately available amino groups in soil solution when green broccoli or young barley was added, the peak was at about 8 days and the levels had returned to about the control level by 14 days after incorporation. Incorporation of old barley caused only a very slight increase in concentration of amino groups.

The ability of P. ultimum to utilize the concentrations of nutrients released into soil solution. Ten ml samples of the third fraction of the rapid cold water extracts were placed in plastic petri dishes. One ml suspensions of *P. ultimum* sporangia and oospores at a concentration of 570 propagules/ml were added. The inoculum was prepared by sonicating an 8-day old culture growing in water from an oatmeal agar nutrient source at 50% power and a tuning of 4 on a Bronwill "Biosonik" sonicator. The sonicated solution was centrifuged three times for 5 minutes at 21.000 *g* at 0°C each time the pellet was resuspended in a sterile cold balanced mineral salt solution with an electrical conductivity of 0.5 millimhos. The density of propagules was counted on a haemocytometer. After four days the amount of growth in each dish was measured. This growth was so small that a photographic method was used to estimate the relative amounts. The dishes were placed on Kodak kodabromide F.5 photographic paper and exposed to light from above. The development of the paper revealed the relative amounts of growth occurring in the different extracts.

P. ultimum was able to grow more in rapid cold water extracts from soil amended with green broccoli or barley than in extracts from soil amended with mature barley or the control unamended soil. All the extracts were made 8 days after incorporation.

Increase in *P. ultimum* in soil contact with green residue.

Incorporation of residue. Salinas fine sandy loam was removed from the field and the following day 200 g lots placed in 7.5 cm deep polystyrene cups. The moisture content of this soil was 19%, a little wetter than the field capacity (18.1%). The surface of the soil was smoothed flat and one layer of cut leaves of rye, (82% M.C.), laid out to cover the surface. The leaf pieces were 1 cm broad and 2.5 cm long and were cut from the three terminal leaves on a number of plants. A layer of the same soil 0.25 inches deep was pressed gently on top of the leaves. Sixteen cups were prepared in this manner and four filled in the same way without the addition of residue. All the cups were incubated in plastic bags to prevent water loss at laboratory temperature, 22°C.

P. ultimum populations. The *P. ultimum* population of the soil, at the time the rye leaf pieces were buried, was determined. Sixteen, 40, 88 and 172 hours after incorporation four cups with residue and one without were treated as follows. The surface 0.25 inch layer of soil was scraped away and discarded and the pieces of leaf carefully removed. On the soil surface below could be seen the imprint of the leaves that were removed. This layer, bearing the imprint, was very carefully scraped from the remainder of the soil with a fine spatula so that no more than 2 mm deep layer was removed. This 2 mm scraping is weighed to make up a 1 g sample which was shaken with 50 ml sterile tap water for 1 hour in a 125 cc flask at 212 r.p.m. The P. ultimum population of the resulting suspension was determined by Stanghellini's method. This experiment was repeated the following week using the same soil which had been stored at 0°C in a plastic bag and a fresh sample of rye (80% M.C.).

The populations of P. ultimum/g soil touching green rye leaves measured in two experiments 16 hours after residue incorporation were lower than the populations in the control soil. Eighty-eight hours after residue incorporation, the P. ultimum populations was 8 or 9 times higher in soil touching green rye leaves.

Soil smears made two hours after green rye leaves had been incorporated showed that sporangia in soil touching residue were already germinating (34 and 42%). After 6 hours 91% of the sporangia in soil touching residue had germinated while only 12% of the sporangia in soil without residue had germinated. The average germination percentages for two experiments are shown in Table 11.

Incorporation of residue and P. ultimum populations in tissue. Rye leaf pieces (81.5% M.C.) were incorporated into Salinas fine sandy loam, (18.5% M.C.) in exactly the same manner as previously described in

Table 11. Percentage of sporangia of P. ultimum germinated in soil touching green rye leaves and in control soil at different times after incorporation of the rye.

	Hours after incorporation					
	2	4	6	8	10	12
Soil touching green rye	38	72	91	87	86	90
Control soil--nothing added	3	5	10	12	7	12

Experiment 1. A total of 80 cups were prepared and 20 were incubated in plastic bags at each of the temperatures 18°C, 24°C, and 30°C in growth chambers and at about 22°C in the laboratory. Five cups were prepared where leaf pieces were buried in the described manner in heat sterilized soil and these were incubated in the laboratory at about 22°C.

Twenty, 44, 68, 116 and 165 hours after incorporation the leaf tissue from four cups at each incubation temperature and from one containing sterilized soil was removed and washed free of mineral particles. The tissue was then immersed in closed vials under freshly prepared 12% Ethanol, 15% sodium hydroxide solution at 60°C for 2 hours. After washing in three changes of tap water and staining in cotton blue the tissue was returned to the vials. Five random samples of leaf tissue were removed from each vial, representing rye decomposing for a certain length of time under certain temperature conditions. A 1 cm^2 piece was cut from each leaf sample with a razor blade. During microscopic examination of the cleared stained leaf pieces a count was made of the number of sporangia and oospores in the tissue.

Table 12 indicates the number of sporangia and oospores of _P. ultimum_ counted in cleared pieces of green rye leaves buried in field soil at 18, 22, 24 and 30°C. Sporangia can be seen after only 44 hours and oospores are seen after 68 hours. The populations of both types of propagules increased most rapidly at 24°C.

Identification of fungus propagules counted in tissue. Because a positive identification in the tissue of the species represented by the sporangia and oospores was not always possible 100 small tissue pieces, selected at random from all the tissue samples before clearing,

Table 12. The number * of sporangia (Sp) and oöspores (Oo) of *P. ultimum* ** per cm^2 of young rye leaf tissue buried 0.25 inch deep in Salines fine sandy loam at different temperatures for different lengths of time.

Temperature	Hours after incorporation of tissue									
	20		44		68		116		165	
	Sp.	Oo.	Sp.	Oo.	Sp.	Oo.	Sp.	Oo.	Sp.	Oo.
18° C	0	0	5	0	14	0	88	112	112	11
22°C lab. temp.	0	0	1	0	10	0	40	86	52	70
24°C	0	0	7	0	45	16	86	178	126	190
30°C	0	0	15	0	35	2	29	35	81	86

* Average of number counted in 5, 1 cm^2 of tissue.

** Positive identification of all propagules was not possible; however, *P. ultimum* was predominantly isolated from the tissue (78% of all isolations).

were plated out on 2% water agar plates. The identity of the Pythium sp. growing from the tissue was then determined by transfer of hyphal tips to rolled oats/water plates and subsequent microscopic examination of the resulting fungus growth.

On the 100 small tissue pieces in which sporangia or oospores were seen and where an identification of the isolated Pythium clones was made on rolled oats agar/water plates, 78% were identified as P. ultimum, 10% were P. splendens and the remainder unidentified spiny oospored-forms.

VIRULENCE OF *RHIZOCTONIA SOLANI* AS AFFECTED BY NUTRITION OF THE PATHOGEN.

The occurrence and severity of a plant disease is dependent upon the interaction of many factors in the physical and biological environment. Several factors, e.g., temperature, moisture, and light, have been investigated extensively, whereas pathogen nutrition has received less attention. That pathogens require a readily available supply of nutrients is an integral part of Garrett's concept of inoculum potential. However, most studies on pathogen nutrition have considered nutrient requirements for growth, sporulation, and spore germination. The quantity and quality of nutrients needed for maximum virulence, and the relative importance of the inoculum, soil solution, and host exudates as sources of nutrients prior to infection, have not been adequately investigated. The influence of the growth medium on the capacity of spores and mycelium to initiate disease, and the effects of supplying nutrients along with the inoculum have been studied, however.

Recent investigations with *Rhizoctonia* showed that after rather short periods in soil, the pathogen becomes less effective in attacking cotton stems. This decrease in virulence may result from a loss of nutrients required to support pathogenic activity. It was the purpose of this study to evaluate the role of pathogen nutrition on virulence of *Rhizoctonia solani* Kühn on cotton seedlings.

Most of this work was conducted using an isolate of *R. solani* (*praticola* type) obtained from diseased cotton growing in the central valley of California. This isolate was also pathogenic to beans, potatoes, and sugar beets. On cotton, numerous thick, dome-shaped infection cushions are formed, followed by tissue maceration.

The nutritional status of the inoculum was varied by growing the fungus on liquid media containing various concentrations of carbon source and/or nitrogen source. The basic medium was composed of 1.75 g KH_2PO_4, 0.75 g $MgSO_4 \cdot 7H_2O$, 37 mg $CaCl_2 \cdot 2H_2O$, 0.8 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $FeCl_3 \cdot 6H_2O$, 0.5 mg $NaMoO \cdot 2H_2O$, 0.9 mg $ZnSO_4 \cdot 7H_2O$, and 0.3 mg $MnSO_4 \cdot H_2O$ in 1 liter of distilled water. Asparagine was added to the basic medium before autoclaving, and glucose was autoclaved separately and added aseptically.

The fungus was grown in still culture on 25 ml medium in 9-cm petri plates for 5 days (unless otherwise specified) at 28°C. Discs of mycelium (2 mm in diam, unless otherwise specified) were then cut from the mycelial mat, washed, and used to inoculate 4- to 5-day-old seedlings of cotton (*Gossypium hirsutum* L. 'Acala 4-42'). Seedlings were grown in a planting mix in the greenhouse. Plants 5 or 6 days old were gently removed and washed. Preliminary work showed that from the time of hypocotyl emergence until seedlings were 9 days old, there was no difference in tissue susceptibility to *R. solani*.

To facilitate observation of disease development, seedlings were placed on glass plates, and the roots were covered with either moistened quartz sand or field soil. A thin layer of this material was placed under the hypocotyl. An inoculum disc was placed alongside each hypocotyl about 1 cm above the transition zone between root and stem, with one edge in contact with the support medium to reduce drying. Aluminum foil was wrapped around each glass plate, enclosing the seedling roots and hypocotyls. The plates were placed in plastic pans containing a layer of water 1 cm deep, and the pans were covered with plastic. Holes were punched in the bottom of the foil to maintain moisture around the plant roots. The pans were maintained in growth chambers under conditions of continuous light and temperature of 28°C. Preliminary studies showed that exposing the cotyledons to light or maintaining the seedlings in darkness had no effect on disease development. However, in light the plants were more vigorous, and saprophytic fungi such as Rhizopus did not attack the cotyledons.

Disease severity was evaluated by measuring the area of macerated tissue.

Relation Between Nutritional Status of Inoculum and Disease Severity.

The influence of nutritional status of inoculum on virulence of R. solani was first studied by diluting the growth medium. The medium containing the basic salts plus 20 g glucose and 2.0 g L-asparagine/liter was diluted to give 75, 50, and 25% of the original concentration. When the pathogen growing on these media was used to inoculate seedlings placed on sand, a straight-line relationship between medium concentration and disease severity was obtained.

In subsequent tests, glucose and asparagine concentrations were varied independently. Inoculation of seedlings with mycelium from media in which the asparagine concentration was held constant at 2 g/liter and in which glucose content varied from 5 to 20 g/liter resulted in a direct relationship between glucose concentration and disease severity. When asparagine content was reduced to 0.5 g/liter, the resulting mycelium was avirulent, regardless of the glucose concentration in the medium.

To further investigate the effect of nitrogen on the production of virulent inoculum, glucose concentration was held constant at 20 g/liter, and asparagine content was varied from 2.0 to 0.5 g/liter. Decreasing the asparagine content of the medium resulted in a corresponding decrease in virulence. A sharp break in virulence was observed between mycelium grown on media with 1.0 and 0.5 g asparagine/liter (Table 13). Because of the distinct difference obtained with glucose at 20 g/liter and 2.0 and 0.5 g asparagine/liter, this system was selected for additional studies. For convenience, medium with 2.0 g asparagine/liter was designated as A, and that with 0.5 g/liter as D.

Several tests were conducted comparing disease severity resulting from inoculation of seedlings with inoculum grown on A and D media. The data from these five experiments with a total of 22 10-seedling replications were combined, and the confidence interval at the 5% level was determined. The mean lesion area 48 to 60 hr after inoculation was 78 ± 6.2 and 7.0 ± 5.5 for seedlings inoculated with mycelium grown on A and D medium, respectively. Typical seedlings 48 hr after inoculation with mycelium grown on A and D are shown. Although the tests were conducted over a period of several months, and conditions varied somewhat from one test to the next, the low variability shows that these results can be readily reproduced.

Table 13. Influence of asparagine concentration on growth of *Rhizoctonia solani* in culture and virulence on cotton hypocotyls.

Asparagine concen- tration a/	Lesion area b/	Dry wt/ culture c/
g/liter	mm^2	mg
2.0	77 ± 5	148 ± 16
1.5	57 ± 4	131 ± 15
1.0	44 ± 2	145 ± 14
0.5	0	143 ± 8

a/ Fungus grown in liquid medium containing macronutrients, micronutrients, and glucose at 20 g/liter.

b/ Each value is the mean of 5 replications, 10 seedlings/replication.

c/ Each value is the mean of 10 flasks.

Four additional isolates of R. solani, all obtained from cotton, were used to compare disease sevcrity from inoculation with mycelium grown on A and D media. In all cases, mycelium from the high asparagine medium was highly virulent, whereas only slight infection occurred with inoculum from the low asparagine medium.

Whenfield soil was used as a support for seedlings on the glass plates, there was an increase in disease over that obtained with quartz sand. This increase was most apparent with inoculum of a low nutritional status. With mycelium grown on A medium, disease severity in the presence of soil was similar to that in sand (x lesion area 57 mm^2). However, with D-grown mycelium, the mean lesion area (6 replications, 10 seedlings/replication) in soil was 23 mm^2 as compared with <1.0 mm^2 when seedlings were supported by sand.

The effect of using inoculum grown on media with lower levels of nitrogen was determined. The fungus was grown on media containing 0.25 and 0.12 g asparagine/liter, and after 5 days, 2-mm discs of mycelium were placed on seedling stems supported by soil. After 48 hr, the mean lesion area (10 seedlings) from mycelium grown with 0.25 and 0.12 g asparagine/liter was 9.7 and 0.4 mm^2, respectively. After 72 hr, lesion size had increased to 11.8 and 4.4 mm^2.

The above tests were repeated several times with similar results. Although the presence of soil increased the severity of disease induced by nutritionally weak inoculum, the importance of the nutritional status in relation to disease severity was readily shown.

Tests were conducted to determine the relationship between studies in the laboratory, greenhouse, and field. Seeds were germinated in field soil in the greenhouse and in a field located at Porterville, California. When the seedlings were 4-5 days old, soil around the hypocotyl was gently removed, a piece of inoculum placed alongside each stem, and the soil replaced. Inoculum consisted of 2-mm discs of mycelium which had grown on both solid (asparagine concentrations below 0.5 g/liter) and liquid media containing 20 g glucose/liter and various levels of asparagine.

In the greenhouse, lesions formed on the hypocotyls, but most of the plants survived the attack. Lesion severity decreased as the nutritional status of the inoculum decreased, due to reduction in the medium, from 2.0 to 0.12 g asparagine/liter.

In the field, inoculum from three different media containing asparagine at rates of 2.0, 0.5, and 0.25 g/liter was used. After 48 hr, a sample of plants was taken to determine lesion severity, and the remainder left to determine plant mortality. Lesion development on the inoculated hypocotyls was related to the nutritional status of the inoculum (Table 14). Stand counts (after thinning) taken 7, 15, and 44 days after inoculation revealed that, although severe cortical lesions had developed on hypocotyls inoculated with the fungus grown on high nutrient medium, the plants were not killed (Table 2).

The results obtained under these conditions in soil were thus comparable to the findings with hypocotyls inoculated on glass plates in the laboratory.

Inoculum Quantity and Disease Severity.

To investigate the influence of inoculum quantity on disease severity, 1-, 2-, and 5-mm-diam discs were taken from 5-day-old colonies

Table 14. Effectiveness of mycelium of *Rhizoctonia solani*, grown on medium containing various concentrations of asparagine, in initiating disease on cotton seedlings growing in the field.

Asparagine concentration	Disease severity after 48 hr		Seedlings surviving/10 ft of row [a]		
			Days after Inoculation		
	Plants	$\bar{x}$ Lesion area	7	14	44 [b]
g/liter	no.	mm^2			
2.0[c]	42	52	39[d]	39	22
0.5	36	33	43	43	22
0.25	44	16	39	39	22
Control (plants not inoculated)	40	4.5	53	53	20

[a] Mean of 2 replications.

[b] Plants thinned prior to this count.

[c] Fungus grown in liquid medium containing macronutrients, micronutrients, and glucose as 20 g/liter.

[d] Lower counts than control due to damage of some plants during removal of soil for inoculation.

growing on A and D media. The mean lesion area, determined from five replications of 10 seedlings each, resulting after 48 hr was 30.6, 50.3, and 101.1 mm^2 from A inoculum, and 5.3, 7.1, adn 22.1 from D inoculum for the 1-, 2-, and 5-mm discs, respectively. With the nutritionally adequate inoculum, the increase in disease severity with increase in inoculum size was more closely related to changes in circumference of the disc than to area or dry wt. The magnitude of the difference between nutritionally adequate and weak inoculum is indicated by the fact that a 1-mm-diam disc of A mycelium produced a larger lesion than a 5-mm disc of D mycelium.

Pathogen Growth in Relation to Inoculum Effectiveness.

Further studies were conducted to determine if differences in virulence brought about by different levels of nutrients were due to an effect on the growth rate of the pathogen.

The relationship between vegetative growth on the various media and function of the mycelium as inoculum was evaluated. Dry wt of the fungal colony at 5 days was not appreciably influenced when asparagine concentration was reduced from 2.0 to 0.5 g/liter (Table 13). When mycelial discs were taken at various distances from the center of 5-day-old cultures on A and D media, there was no relationship between dry wt of discs and their effectiveness as inoculum.

A second aspect of the relationship between growth and virulence involves the development of hyphae from the inoculum following inoculation. To obtain some indication of the ability of the fungus to grow from inoculum discs, the rate of growth on 1.5% Oxoid-agar agar No. 3 in petri plates at 28°C was determined. The rate of growth from a 2-mm-diam mycelial disc from D. medium was slower than that from mycelium from A medium for the first 12 hr (x colony radius of 6.4 for A and 2.5 for D). However, for the next 40 hr, the growth rate of mycelium from discs taken from the two media was similar (approximately 0.7 mm/hr). After 52 hr, the mean radius was 33.5 and 28.0 mm for colonies originating from A and D media inoculum discs, respectively.

Growth from the two types of inoculum was compared by determining the density of mycelium formed on the purified agar. Two-mm mycelial discs were placed on a thin layer of agar deposited on a microscope slide. The slides were incubated at 28°C, and after 11 and 22 hr, the number of hyphal tips in a randomly selected microscope field (X400 magnification) was counted. The mean number of tips per field (20 fields counted) from mycelium derived from A and D discs was 10.2 and 9.1 after 11 hr, and 17.6 and 14.9 after 22 hr, respectively.

The ability of the pathogen to grow from inoculum discs onto seedling stems and the rate of disease development was determined. Seedlings were inoculated with fungus grown on A and D media containing 20 g/liter glucose, and maintained as previously described. After 24, 48, and 72 hr, seedlings were sprayed with 0.1% trypan blue dissolved in 10% acetic acid to avoid disturbing the hyphae, and the growth of the pathogen on the stems was measured. Lesion development was also determined.

During the first 24 hr, growth from the D inoculum lagged about 12 hr behind that from the A inoculum. From 24 to 48 hr, the growth rate of the fungus from the two inoculum sources was approximately equal

(0.37 and 0.5 mm/hr for A and D inoculum, respectively). Between 48 and 72 hr, growth declined, presumably because of increased moisture stress near the top of the aluminum foil envelope.

Growth of the pathogen on cotton hypocotyls was followed closely by the formation of infection cushions. Twenty-four hr after inoculation with mycelium from A medium, numerous infection cushions had formed, whereas from D medium mycelium there was hyphal branching, but cushions had not formed. In 42 to 48 hr, infection cushions had formed on hypocotyls inoculated with mycelium from both A and D media.

A comparison of pathogen growth on hypocotyls and lesion severity resulting from A and D inoculum revealed an interesting relationship. After 24 and 48 hr, growth from the D inoculum lagged 12 and 5 hr, respectively, behind that from the A inoculum. However, after 48 and 72 hr, lesion development resulting from inoculation with mycelium grown on D medium lagged 19 and 31 hr, respectively, behind that from inoculum grown on A medium. This indicates that the differences in disease severity were not due entirely to differences in rate of hyphal growth.

INVESTIGATION OF THE MECHANISM OF GREEN MANURE INFLUENCE ON COMMON SCAB OF POTATO.

A long-term study in the San Joaquin Valley of California revealed that cover cropping with incorporation of the green plant residue exerted a dramatic effect on the build-up of common scab of potato. The results of this study can be summarized as follows: Under continuous potato culture without any cover crop or green manure the incidence of potato scab increased steadily, reaching a maximum after 8 years. A barley cover crop and green manure increased scab incidence to a level nearly twice that of the no cover crop control. A soybean cover crop and green manure completely prevented the build-up of potato scab. Although a soybean cover crop prevented disease build-up it was completely ineffective in reducing scab incidence once the pathogen was well established.

Because of these striking results, investigations concerning possible mechanisms which could be responsible for the influence of these green manures in potato scab were undertaken. The low incidence of disease at the beginning of the test suggested a low population of the scab organism in the soil. The disease build-up in the control and barley plots was indicative of an increase of S. scabies in this soil. The absence of disease increase in the soybean plots could be due to a lack of pathogen population increase or to an interference with the pathogenic activities of the scab organism. Two lines of evidence obtained from the field plots indicate that the soybean amendment prevented an increase in the population of S. scabies: (i) Soybeans were not effective in reducing disease in plots where scab incidence was high; and (ii) when the soybeans were omitted for three consecutive years, the incidence of disease did not increase. If the population of S. scabies had increased in the soybean plots, then a cessation of this cropping practice should have resulted in the occurrence of the disease at a level corresponding to the population of the pathogens present in the soil. The fact that disease did not occur in these plots, indicated that the population had remained at a low level. Therefore, although no direct evidence could be obtained because of the difficulty of quantitatively assaying soil for S. scabies, it was assumed that the green manures were affecting pathogen population.

The incorporation of large amounts of fresh plant material into soil would provide abundant substrate for organisms antagonistic to S. scabies. The role of antagonism in biological control has been considered for many soil-borne plant pathogens including S. scabies.

This phase of the report presents the results of investigations into the prevalence and activity of organisms antagonistic to S. scabies and their possible role in the regulation of population levels of the scab organism in soil.

Isolation of Bacteria Antagonistic to S. scabies and Non-Pathogenic Streptomyces spp.

A standard plating technique was used to assay soil from the field plots for organisms antagonistic to S. scabies. In this procedure, spores of S. scabies were suspended in the high dextrose nutrient agar. The medium was held at 52°C and poured into petri plates containing spores of the pathogen. After cooling, 0.1 ml of an appropriate soil dilution was spread evenly over the surface. The plates were placed in a 45°C oven with lids partially open for 4 hr to remove excess water followed by incubation at 31°C. The organisms which could inhibit *S. scabies*, when growing on this medium, formed clear plaques in the otherwise heavy growth of the scab pathogen.

Those organisms which produced plaques were transferred to PDA and tested individually. Testing procedures included streaking the antagonists on agar medium and opposing these with perpendicular streaks of the test organisms. We also grew the antagonists in a liquid medium which was subsequently filter sterilized and tested with a standard filter paper disc antibiotic assay technique.

Soil from the Shafter plots was assayed for antagonistic organisms at several times of the year (Table 15). This covered a period from approximately 2 weeks after soybeans were turned under, through growth and turning under of barley (early December), to midway into the potato growing season.

From all of the plots at all sampling times, one type of bacterium was the predominant antagonistic organism which was isolated. The bacterium was a gram positive, motile, spore-forming rod. On potato-dextrose agar slants the organism appeared as a rapidly spreading, dull, cream-colored to light brown colony with a wrinkled surface. Following Smith, Gordon and Clark, the bacterium was keyed out as being *Bacillus subtilis* or *B. licheniformis*. Because of the difficulty in distinguishing between these species, no further classification was attempted. For convenience, the organism will subsequently be referred to as *B. subtilis*.

Table 15. Population of bacteria antagonistic to S. scabies in field plots in which various crop residues have been incorporated. Antagonists/g of soil x 10^3

Crop incorporated	Date of sampling				
	11/1/60*	12/1/60**	2/14/61†	4/25/61††	$\bar{x}$
Barley	108	138	280	144	167
Soybean	144	142	156	276	179
No amendment	180	138	220	172	177

* 1 replication; 3 plates/replication

** 3 replications; 6 plates/replication

† 3 replications; 9 plates/replication

†† 3 replications; 10 plates/replication

Other antagonistic bacteria, including some *Streptomyces* spp, were obtained, but these occurred less frequently. A large number of antagonists were transferred from assay plates to PDA for observation. Of the 131 transferred, 75 percent were similar in appearance to the isolates identified as being of the *B. subtilis* type. Twelve of these isolates were tested for antagonism and found to be highly active against *S. scabies*.

The population of antagonists in the plots varied from 108 to 280 x 10^3 units/gram of soil. Throughout the four samplings, there was no pattern to suggest a consistent difference in antagonist count among plots receiving soybean or barley green manure amendments or those which were planted continuously to potatoes in the absence of cover crop or amendment (Table 1).

The occurrence of organisms antagonistic to non-pathogenic *Streptomyces* spp. was also determined. Five representative non-pathogens were used as test organisms. *Streptomyces scabies* was included in the test as a control. With three of the non-pathogenic isolates no antagonists were obtained. In the control plates containing *S. scabies*, an average of four antagonists/plate were obtained. Approximately one antagonist/plate was recovered with the other two non-pathogenic isolates, and these were similar to the bacteria which were predominant on the control plates. This supports the observation that bacteria of the *Bacillus subtilis* type are a common antagonist of *Streptomycetes* and suggests that *S. scabies* is more sensitive to the activities of this bacterium than are *Streptomyces* spp non-pathogenic to potato.

Activity of *B. subtilis* Type Bacteria Against *S. scabies* and Non-Pathogenic *Streptomyces* spp.

Several representative isolates of bacteria were selected for further testing, based on their activity against a standard *S. scabies* isolate. These were tested against 16 isolates of *S. scabies* and 18 isolates of non-pathogenic *Streptomyces* spp using nutrient agar streak plates. The isolates of *S. scabies* used in this study were obtained not only from potato growing areas in California, but from several other states*. These isolates were tested for pathogenicity in the greenhouse and maintained in the laboratory on potato-dextrose-agar. The isolates of bacteria varied in their antibiotic activity, however, the pathogens were consistently inhibited to a greater extent than the non-pathogens (Table 16). Data presented in Table 2 was obtained from 3 experiments using a single isolate of *B. subtilis* which was typical of those isolated from the plots. Tests with other isolates provided similar results.

The activity of the bacteria on various substrates and their specificity to *S. scabies* was further tested by growing them in liquid medium which was subsequently filter sterilized and assayed. Media tested included potato dextrose broth (PDB) nutrient broth, Patrick's high dextrose broth (PHDB), and water extracts of soybean and barley tissue. Nutrient broth proved to be a relatively poor substrate while PDB and PHDB supported good antibiotic production. The plant tissue extracts

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*Appreciation is expressed to Dr. J. Menzies for providing *S. scabies* isolates from states other than California.

Table 16. Sensitivity of S. scabies and non-pathogenic Streptomyces spp. to bacitracin and the antibiotic produced by B. subtilis isolated from Shafter field plots. Relative inhibition based on sensitivity of standard S. scabies isolate*.

S. scabies			Non-pathogenic Streptomyces spp.		
Isolate no.	Antibiotic**	Bacitracin†	Isolate no.	Antibiotic**	Bacitracin†
115 (Standard)	100	100	80	112	85
117	100	57	98	59	190
116	100	86	111	50	57
120R	74	100	62	34	17
105	58	74	70	30	50
130D	58	109	45	25	0
130B	58	166	57	23	0
114R	50	50	1	23	0
130C	50	57	35	19	0
121R	50	57	35	19	0
130A	50	150	101	17	17
121R	50	74	10	17	0
130A	50	150	101	17	17
B19R	39	65	131	11	25
128R	39	65	65	11	50
127	30	74	74	11	65
			58	0	28
			106	0	33
$\bar{x}$ Inhibition	61	74	$\bar{x}$ Inhibition	28	43

* Inhibition relative to that of the standard taken as 100. Table compiled from data of 3 experiments.

** A 24-h soybean extract culture with 3.10 units activity was used.

† A 40-ppm solution of bacitracin with 2.10 units activity was used.

were also good substrates for the antibiotic production, soybean being better than barley. More information on this substrate effect will be presented below. The antibiotic produced on all substrates tested was consistently more active against S. scabies than the non-pathogens.

Characterization of the active substance. The active compound produced by B. subtilis type bacteria isolated from the Shafter plots had the following characteristics: (i) elaborated into the medium and diffused readily in 2% agar; (ii) non-dialyzable against cold running water; (iii) not extracted with ether, chloroform or acetone but extracted with 1-butanol with some loss of activity; (iv) not precipitated by lowering or raising pH. The above characteristics indicate that the antibiotic is similar to bacitracin which has been reported to be produced by B. subtilis and related species. In addition, S. scabies was much more sensitive to bacitracin than non-pathogenic Streptomyces sp., presenting a pattern similar to that obtained with the unidentified antibiotic (Table 16). However, when comparing individual isolates of S. scabies and Streptomyces spp., it was apparent that the activity spectrum of bacitracin and the unknown were different. Therefore, although the unidentified antibiotic possesses characteristics which suggest that it belongs to the same group of antibiotics as bacitracin it is not identical to bacitracin.

Antagonist Population During Decomposition of Plant Residue in Soil.

The influence of crop residues on plant pathogens in soil must, in part, occur during decomposition of these amendments. Therefore, if the population of bacteria antagonistic to S. scabies is to be implicated as a factor in the effects obtained when certain plant material is incorporated into soil, soybean plant material, which has beneficial effects, might be expected to support a higher population of antagonists during decomposition than non-beneficial barley amendments. The build-up of antagonists during decomposition of barley and soybean tissue in soil was studied under greenhouse conditions.

Soil from the field plots (Hisperia sandy loam) was mixed 1 part soil and 1 part sand and maintained at or just below field capacity, in soil columns 16 inches in diameter and 36 inches high. Temperature during decomposition was 20-23°C. One gram of tissue (wet weight) and 5 g soil were placed in bags of 80 mesh nylon and buried at a depth of 6 inches. At intervals, bags were recovered and the contents suspended in water and the antagonist population assayed by the dilution plate technique. Packets containing 5 g of soil without tissue were included as a control.

These tests showed that the antagonistic bacteria increase in number when plant material is present in soil (Table 17). After 4 days the population of antagonists in bags containing soybean and barley tissue was 3 to 4 times that in the bags without tissue. The number of antagonists remained high in the tissue bags for 21 days. After 28 days the population had decreased to the same point as the soil alone. There was no consistent difference between barley and soybean in supporting growth of the antagonists. These reports, supported by isolation from the field plots (Table 15), suggest that if soil amendments operate through

Table 17. Changes in population of bacteria antagonistic to *S. scabies* during decomposition of barley and soybean tissue in soil. Antagonists/g dry soil (x 10^3). Values are the mean of 3 experiments with 3 replications/sampling period.

Treatment	Period tissue was in soil (days)				
	4	7	14	21	28
Soil only	10.9± 2.3	10.6±2.3	22.8±5.7	16.5±11.7	9.9±4.0
Soil + soybean tissue	43.9±12.2	31.0±4.0	32.0±4.8	42.9± 2.4	6.6±2.0
Soil + barley tissue	30.7±12.3	32.3±2.4	30.7±7.8	29.7± 1.0	8.6±4.5

their affects on certain antagonistic bacteria, this influence must involve more than changes in antagonist population.

Plant Material as a Substrate from Antibiotic Production by *Bacillus subtilis* Type Bacteria.

Although there was no difference between barley and soybean tissue as a substrate in soil for growth of bacteria antagonistic to *S. scabies*, there was an appreciable increase in antagonist population with both tissue over non-amended soil. This raised a question concerning the influence of these materials on the quantity of antibiotic substance produced. To investigate this aspect, water extracts of fresh and partially decomposed tissue were used as substrates for bacterial growth and antibiotic production.

Tissue was cut into small pieces, added to water in a ratio of 100 g (wet weight) tissue to 800 ml water, macerated for 5 minutes in a waring blender, and heated for 15 minutes at 90°C. The residue was removed by filtration and the filtrate diluted with water, dispensed 5 ml per 25-ml Erlenmeyer flask and autoclaved. Extracts were diluted to obtain various levels of bacterial growth. A comparison with millipore filter sterilized extracts did not indicate any adverse effect produced by autoclaving. The easier procedure was therefore adopted. The various extract dilutions were seeded with 1 drop (.05 ml) of a 24-hour broth culture of the bacterium and incubated at 28°C on a reciprocal water bath shaker. After 24 hours growth, the bacteria were removed by millipore filtration and the sterile filtrate tested for antibiotic activity using a filter paper disc assay technique. Bacterial growth was determined by direct count using a counting chamber. The suspensions were briefly sonicated prior to counting to disperse chains of bacteria.

To have a uniform basis for comparison and to obtain quantitative data, the activity of test solutions against S. scabies was related to activity of commercial bacitracin. Bacitracin was selected because of its similarity in activity and properties to the substance produced by our antagonistic bacteria. A standard isolate of S. scabies (No. 115) was selected and the relationship between concentration of bacitracin and diameter of the inhibition zone was determined. A straight-line relationship between the log of bacitracin concentration and zone diameter was obtained from 10-1000 ppm against the standard isolate of S. scabies. Five units is therefore equivalent to the activity of 100 ppm bacitracin.

When antibiotic production, based on bacitracin unit equivalents, was plotted against bacterial growth, there was consistently 2 to 3 times as much antibiotic produced with soybean tissue extract as a substrate than with barley. This effect held over a temperature range of 21 to 30°C, and with 4 isolates of bacteria which were tested. At the lower temperature, the rate of bacterial growth and antibiotic production was reduced. The difference in quantity of antibiotic produced between soybean and barley tissue as substrates was also apparent when extracts of partially decomposed tissue was used. After 7 days in moist soil, the tissue no longer contained sufficient growth for antibiotic production. An analysis for total carbohydrates using anthrone reagent revealed that these compounds were essentially absent at this time.

Lack of Demonstrable Antibiotic Activity in Extracts of Tissue Decomposing in Soil.

Repeated attempts to demonstrate antibiotic activity in water extracts of soybean or barley tissue decomposing in soil were unsuccessful. Studies were conducted to determine whether the antibiotic substance was absorbed by soil particles. Highly active culture filtrates and solutions of commercial bacitracin were added to the top of (1.5 x 10.0 cm) columns of field plot soil and several recovery procedures tested. Alcohol displacement and elution with water or phosphate buffer of varying concentration and pH all failed to remove the antibiotic from the soil. This indicates that inability to demonstrate antibiotic activity in extracts of tissue from soil does not necessarily mean that the antibiotic was not produced.

DISCUSSION AND SUMMARY

The investigation demonstrated that substances possessing phytotoxic properties may be formed during the decomposition of plant residues under natural conditions in the field. Such substances are most likely formed during the early stages of the decomposition process, are relatively ephemeral, and are inactivated during subsequent decomposition. Whether the toxic compounds were synthesized by soil microorganisms using plant material as substrate or were the breakdown products inherent in the plant tissues was not determined. The results with broadbean, broccoli, barley, and rye suggest, however, that in many instances they are probably a combination of both.

In these studies, phytotoxic compounds of apparently significant potency were detectable only after the decomposing plant residue had been freed from most of the adhering soil prior to extraction and only up to a certain stage of decomposition. In analogous studies of antibiosis, other investigators found it impossible to detect antibiotic activity in extracts of natural soil. Significant concentrations of such substances could be detected, however, in the microenvironment of suitable substrates such as plant debris. These various results may not only explain discrepancies in the relevant literature concerning the production and existence of such substances, but also indicate a more reliable procedure in studies of this kind, namely to concentrate directly on the loci of greatest potential by separating plant fragments from the soil and working only with them.

Some of the conditions of prime importance in the production of phytotoxic compounds during plant-residue decomposition have been described. Further, depending on the decomposition conditions, substances highly toxic, nontoxic, or stimulatory to plants can be formed during the decomposition of similar plant residues. Because of the ephemeral nature of such products and their relatively rapid transition from one type of physiological activity to another, their occurrence may frequently be missed. It is evident, too, because of inactivation, absorption, or masking by the greatly preponderant soil mass, such substances might be difficult to detect and assay. Thus, in dealing with the question of phytoxicity of plant residue decomposition products, their presence, production, and biological activity, attention should be directed primarily to the loci where production of such substances is most likely, the conditions that lead to their formation, and the microhabitat of the root where effective activity of such compounds is likely.

Diffusion of phytotoxic substances from the loci of production appears limited and effective concentrations of such substances could only be detected in the decomposing plant-residue fragments and the soil in contact with such fragments. Thus, the extent of root injury and the total effect on plants would depend primarily on the frequency of chance encounters of the growing root system with plant-residue fragments when the accompanying decomposition products are toxic. The possible exception would be in instances where abnormally large quantities of plant residue had been incorporated with the soil. In such instances, effects might be widespread and severe if conditions for decomposition were favorable for formation of phytotoxins. In most fields, however, because of uneven and relatively sparse distribution of plant debris, the injurious effects on plants would be limited to chance encounters of roots with loci of toxin formation and effective concentration.

The effect of phytotoxic substances with which these studies are concerned is difficult to evaluate under field conditions. Nevertheless, there is sufficient evidence to suggest that involvement in the etiology of root disease is one of their most important roles. In addition to their direct toxic action, they apparently so condition root tissues as to predispose them to invasion by the various low-grade pathogens. The toxins can affect whole roots or segments of roots and pave the way for invasion by the various pathogens which require some sort of infection court such as lesions or moribund tissue before they can become a factor in root pathogenesis.

The experiments indicate that severity of bean root diseases caused by *F. solani f. phaseoli*, *T. basicola*, and *R. solani* may be increased considerably when roots are exposed to toxic extracts obtained from decomposing plant residues.

The greenhouse experiments were carried out with concentrations of inoculum and of extract that were perhaps higher than normally found in nature. Possibly, the increased damage to the root system might be the result of the additive effect of toxic extracts and pathogens acting independently. The results obtained with *R. solani*, however, are of particular interest and reveal perhaps another role of toxic extracts in nature. In this case, the fungus actively invaded the plant only after roots had been exposed to the toxic extract. The major role of the extract, therefore, apparently was to precondition or predispose roots to fungus invasion. Perhaps, this is a major role of these toxic compounds in root rot etiology in nature. As more knowledge about root rots is obtained, the demarcation line between pathogens and saprophytes becomes increasingly indistinct. Apparently also, certain root rots may not be caused by a specific pathogen, but in many cases may be due to organisms that ordinarily cause little damage; but which are able, when conditions are favorable, to become suddenly more aggressive. Among soil factors most likely to cause this shift in behavior could be conditions influencing the production of toxic products during decomposition of plant residues. The formation of these compounds and their weakening effect on the plant root may be all that is necessary to trigger the dormant pathogenic activity of a number of organisms lying in the vicinity of the root and leading to a root rot attributed to a "complex" rather than to a specific pathogen.

The experiments have shown that activity of the extracts, at least in part, weakens and alters permeability of host cells; or kills them. Thus many substances (including ninhydrin-positive compounds) diffuse out from the plant tissues. Possibly, also, the extracts contain unknown substances, perhaps nutritionally (or otherwise) favorable to pathogenic development of the fungus. This model probably does not hold true for all root fungi. In some cases, death of host cells through direct toxic action of decomposition products would be the primary function of such compounds, particularly in the case of weak or low-grade pathogens. The latter are often associated with certain root disorders requiring necrotic tissue for initiation of pathogenesis.

The results presented here show that toxic products need not necessarily be formed by the pathogen growing saprophytically on the host or in the soil on an organic foot base; but can be derived independently from crop residue decomposition. As a consequence, an invasion by a given organism possibly cannot always be directly correlated with its own capacity for toxin production. Perhaps, also, the pathogen need not become established solely through its own efforts. The role of toxins in root infection should not, therefore, be considered the sole province of the pathogen, because phytotoxic substances from other sources in the soil may be important in infection.

Crop residues are important in the control of Fusarium, Rhizoctonia, and Thielaviopsis root rots of bean. This perhaps is due to a tying up of available nitrogen in the soil by organisms decomposing the residue. The pathogen under such circumstances is deprived of this needed food base and cannot attack the plant. The results obtained here show that crop residues, having a suppressive effect on a given pathogen, also can enhance invasive capacity of this same pathogen. The differences in behavior thus elicited apparently are dependent on current, ecological environmental factors.

The results reported here, therefore, may have important implications with respect to mechanisms of resistance and susceptibility of plant roots to soil-borne pathogens and various basic aspects of invasion and pathogenesis mechanisms.

Detailed investigation of the role of crop residues as a factor affecting seedling diseases of lettuce indicated that seedlings were damaged when concentrations of phytotoxins were insuffieient to be directly responsible. It was shown that their damage was due to attack by Pythium ultimum. It was further shown that crop residues dramatically increased the population of P. ultimum in the soil. The increase in population was associated with an increase in damage to lettuce seedlings.

It was also shown that organic residues released soluble nutrients into the soil and caused an increase in bacteria, actinomycetes and fungi. The increase of these saprophytes was associated with the decrease of P. ultimum populations. The influence of crop residues on P. ultimum populations appear to involve an interaction between the utilization of the residue as a substrate for P. ultimum and the competitive activities of the other organisms involved in the residue decomposition.

R. solani survives in field soil for extended periods of time, and attacks seedlings when conditions are favorable. For infection to occur, the propagules of the fungus must germinate or resume growth. Hyphae must then grow to the host, form infection structures, penetrate the epidermis, and establish themselves in the host. These processes require energy and the synthesis of new structural materials. Only three sources are available to provide the required nutrients: (i) propagules of the fungus; (ii) soil solution and organic matter in soil; and (iii) exudates from the host plant. If the quantity or quality of available nutrients were inadequate, fungal activity and probability of infection would be correspondingly reduced. It is, therefore, necessary to understand the nutritional requirements and relationships leading to infection.

The present study has provided data that demonstrate the importance of pathogen nutrition in pathogenesis by R. solani. The nutrient requirement for maximum virulence of R. solani was found to be greater than for vegetative growth. This emphasizes that when evaluating the pathogenic capabilities of R. solani, the nutritional status of the inoculum must be taken into account. Criteria such as dry wt or volume of inoculum are not necessarily adequate, because the fungus can make satisfactory vegetative growth on a medium, yet the mycelium will exhibit a degree of virulence far below the potential of the fungus.

Studies on other fungi have shown that nutrients present in the infection court can influence pathogenesis. All of this work emphasizes that pathogens can take up and utilize nutrients prior to establishment within a host plant.

The use of field soil as a support for the seedling hypocotyls resulted in increased virulence of mycelium grown on a medium low in asparagine, suggesting that the pathogen may be obtaining supplemental nutrients from the soil. R. solani can rapidly increase in virulence by utilizing nutrients from an external source.

In view of the marked influence of nutrition on virulence of R. solani, altering the nutritional status of the pathogen as it exists in soil or influencing the nutrients available to the pathogen in such a way that disease is avoided appears possible. In fact, beneficial cultural practices such as rotation may be due to effects on pathogen nutrition rather than to a reduction in pathogen population.

There are several reports which suggest that organisms antagonistic to S. scabies may play a role in the effects of soil amendments upon the population of this pathogen. This work, in addition to the frequent references which implicate antagonists as a factor influencing the behavior of soil-borne pathogens prompted our assay of the field plot soil for organisms inhibitory to S. scabies. It was found that bacteria of the Bacillus subtilis type were the predominant antagonists isolated by the method employed. This was not surprising in view of the numerous reports of these bacteria as microbial antagonists. However, this group of bacteria has received little attention as antagonists of S. scabies.

When these antagonistic bacteria were tested for activity against isolates of S. scabies from various parts of California and from other states, as well as numerous isolates of non-pathogenic Streptomyces spp., an interesting relationship was revealed. The isolates of S. scabies were consistently more sensitive to the antagonists than the non-pathogens. This phenomenon suggested that the possibility of antagonistic bacteria influencing the population of S. scabies in soil should receive more attention.

An assay of the field plots at various times of the year did not reveal any consistent pattern with respect to antagonist population and prior green manure treatment. Since the influence of the soil amendments probably is exerted during their decomposition, the change in antagonist population during breakdown of soybean and barley tissue in soil was investigated. The number of antagonists increased rapidly when fresh plant material was buried in soil but no difference could be detected between barley and soybean tissue as a substrate for increase of the bacterial population.

The next phase of the study was to compare barley and soybean tissue as substrates for antibiotic production by the antagonistic bacteria. Here a striking difference was observed. Each bacterium, when growing on a water extract of soybean tissue produced 2 to 3 times as much antibiotic as when growing on a similar extract of barley tissue. Soybean was found to be an excellent substrate for production of antibiotics by bacteria of the B. subtilis group. Therefore, although both plant materials were equally suitable as substrates for bacterial growth, antibiotic production was much greater on soybean extracts than on barley extracts.

An evaluation of the results of this study reveals points which suggest that bacteria antagonistic to S. scabies may play a role in the effects of green manures on the build-up of potato scab in field soil: 1) bacteria strongly antagonistic to S. scabies are present in relatively high populations in the soil (108 to 280 x 10^3/g of soil); 2) S. scabies is much more sensitive to these bacteria than the majority of non-pathogenic Streptomyces spp.; 3) when barley and soybean tissue is incorporated into the soil, the population of antagonists increases rapidly indicating growth on these substrates; 4) soybean tissue is a much better (2 to 3 x) substrate for antibiotic production than barley tissue.

Considering the above information, it may be visualized that the addition of soybean tissue to soil functions to maintain *S. scabies* at a low level of infestation by serving as an excellent substrate for antibiotic production by soil bacteria. During decomposition of the soybean tissue, the population of *S. scabies* might be maintained at the existing level or reduced somewhat, to counteract a slight build-up on the few infected tubers which occurred the previous season, by a combination of lethal effects and/or a reduction in its ability to compete for available nutrients with *Streptomyces* spp. tolerant of the antibiotic. If such an effect does occur, it must be subtle because field results show that soybean amendments will prevent disease build-up but will not control the disease in heavily infested soil. Conversely, when barley is used as a green manure there may be insufficient antibiotic production to prevent a build-up in the population of *S. scabies*.

Although the data obtained in this study would support the suggestion that antagonist bacteria may be instrumental in the effects of green manures on potato scab, it must be recognized that direct evidence concerning their importance is lacking. For example, it was not possible to demonstrate the presence of antibiotic activity in aqueous extracts of tissue decomposing in soil. However, since antibiotic added to soil could not be recovered, this inability to demonstrate antibiotic production under natural conditions does not mean it did not occur. Even if it is assumed that the antibiotic is produced during tissue decomposition in soil, there remain problems of distribution and effective concentration. A major obstacle to gaining a more complete understanding of the mechanisms by which green manures influence the build-up of potato scab is our incomplete knowledge concerning the behavior of S. scabies in soil.

Results of this study show that when investigating the significance of antagonistic organisms in the ecology of plant pathogens, attention should be given to the possibility that the pathogen may be more sensitive to the antibiotic produced by the antagonist than non-pathogen members of the genus. Under certain conditions this could provide a competitive advantage to the non-pathogen. Furthermore, consideration of antagonist populations alone may be an inadequate indication of their importance because quantity of antibiotic substances produced may be dependent not on numbers of the organism, but on the nature of the substrate on which the antagonist grows.

In summary, it is evident that the biological consequences of decomposition of plant residues in soil are immense and most phases of plant development are affected.

SUMMARY

1. Substances possessing phytotoxic properties may be formed during the decomposition of a variety of plant residues in the laboratory and under natural conditions in the field.

2. Sequential studies showed that the degree of phytotoxicity depended on the length of the decomposition period. Little or no phytotoxicity was present soon after the crops were turned under. The level of potency gradually increased until a maximum was reached at 15-25 days decomposition. A steady decrease in potency followed until, by 30-45 days, little or no phytotoxicity remained.

3. Green plant residues in the early stages of decomposition are more phytotoxic than mature crop residues. With the latter the phytotoxicity is evident in the later stages of decomposition and is maintained for longer periods.

4. Major factors determining phytotoxicity were temperature and moisture. Cold, wet soils were generally favourable to the development of phytotoxins, whereas in warm, well aerated soils, little or no phytotoxicity developed. This is predominantly a reflection of aerobic and anaerobic conditions in the soil, types of organisms favored by such conditions and level of supply of decomposable organic matter.

5. A wide range of injurious effects on plants was induced by the phytotoxic decomposition products. These included delay or complete inhibition of seed germination, stunted over-all plant growth, injury to the

root system, deranged nutrient absorption, chlorosis, wilting and killing of plants.

6. Roots of plants appeared to be highly sensitive to the phytotoxins. The primary root appeared especially sensitive. The apical meristem region of the root often developed abnormally, became discolored and necrotic. Injury to the apical meristem was a consistent and characteristic effect of phytotoxins on roots and served as a useful diagnostic expression of phytotoxin injury in the field.

7. The phytotoxins also had an inhibiting effect on respiration of root tips and of seedlings and growth of root hairs was inhibited. There was injury in the form of depressions and discolorations when drops of toxic extracts were placed on the surface of plant roots.

8. The phytotoxins also appeared to affect the permeability of plant cells which resulted in a marked increase in exudation of ninhydrin-positive substances and sugars from areas in contact with the toxins. These may play significant roles in plant nutrition and root disease etiology.

9. In field and laboratory experiments phytotoxins of significant potency were only detectable if the decomposing plant residues had been freed of most of the adhering soil prior to extraction with water.

10. Inclusions of large amounts of soil in the extraction procedure diluted the concentration of the phytotoxins to a level where they could not be detected by the conventional bioassays. This finding could explain why

the detection of phytotoxins in the field had been so difficult to accomplish in the past even though their existence has long been suspected.

11. Based on the extensive experiments it is evident that concentration of phytotoxins in soil and their effects on plants should also be considered from a microecological aspect. The extent of root injury or other effects on plants is primarily dependent on the frequency of chance encounters of a growing root system with fragments of plant residue at a time when decomposition products are toxic.

12. When abnormally large quantities of plant residues are added to the soil, the phytotoxic effects are widespread only if the decomposition conditions are such that results in toxin formation. Phytotoxic substances are produced most abundantly in the early stages of decomposition and under anaerobic conditions.

13. Two major components were noted in the phytotoxic extracts. The first is water soluble but not ether soluble. It is probably made up of salts which accumulate in plant tissues during growth. This problem can become serious in intensively cultivated semi-arid areas of the West.

14. The second fraction is soluble in water and in ether and contains the phytotoxins proper.

15. Extraction of barley residues in ether at pH 2.0 produced a fraction that accounted for 60% of the total phytotoxicity of the extracts.

16. On the basis of bioassays, paper chromatography and gas chromatography, the phytotoxins were identified as benzoic acid, phenylacetic acid, 3-phenylpropionic acid and 4-phenylbutyric acid. There were other active zones which were not identified. Many of these substances have not heretofore been reported as products of crop residue decomposition.

17. Benzoic acid and phenylacetic acid were also found in barley residues that had decomposed under natural conditions in the field and in phytotoxic extracts of cotton, cowpea and soybean. Various amino acids and many other intermediate breakdown products were also found.

18. Studies on successions of microorganisms involved in decomposition of green barley and phytotoxin production revealed a marked enrichment of anaerobic bacteria and the dominance of _Geotrichum_ and yeasts in laboratory experiments.

19. Of the various organisms isolated from decomposing crop residues, the yeast isolates increased the concentrations of benzoic and phenyl acetic acids while _Clostridium_ ssp. and _Geotrichum_ sp. increased the concentration of hydrocinnamic and phenylbutyric acids. The concentrations of phenyl acids found after 21 days of degradation by only one organism were not as great as those found when the degradation was being affected by a range of microorganisms naturally selected from soil.

20. Studies were conducted to determine the relationship of crop derived phytotoxins to soil-borne diseases. Root rot of bean caused by _Fusarium solani_ f. sp. _phaseoli_, _Thielaviopsis basicola_ or _Rhizoctonia solani_

was considerably increased in the presence of phytotoxic substances.

21. Relatively low concentrations of phytotoxins, concentrations that did not produce visible damage to the host, increased disease severity considerably when any of the above pathogens were added to the roots.

22. It was also found that the phytotoxic compounds appeared to alter the plant's resistance mechanisms so that varieties that were resistant became susceptible. This was clearly illustrated with root rot disease of cotton and *T. basicola*.

23. The important mechanisms operating in the host-pathogen interaction complex were; direct injury to host cells, increased exudation from host cells, and stimulation of germination of resting propagules of the pathogens.

24. In the case of *Pythium ultimum* the role of phytotoxins in the root rot complex of cotton was not as evident. The experiments suggested that populations of *P. ultimum* were considerably increased by the various cover crops and the resulting decomposition products. *P. ultimum* populations ranged from 42 propagules/g. soil, these increased to 7460 propagules/g. soil in the presence of green crop residues.

25. Experiments also showed that crop residues are important in the control of certain root diseases. The studies revealed that pathogenesis of *Rhizonctonia solani* is affected by available nutrients. Important sources of available nutrients are crop residues and their decomposition products.

26. Crop residues may serve as substrates for bacteria that are antagonistic to soil-borne plant pathogens. This was suggested as a possible explanation for the effect of green manures on common scab of potato.

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